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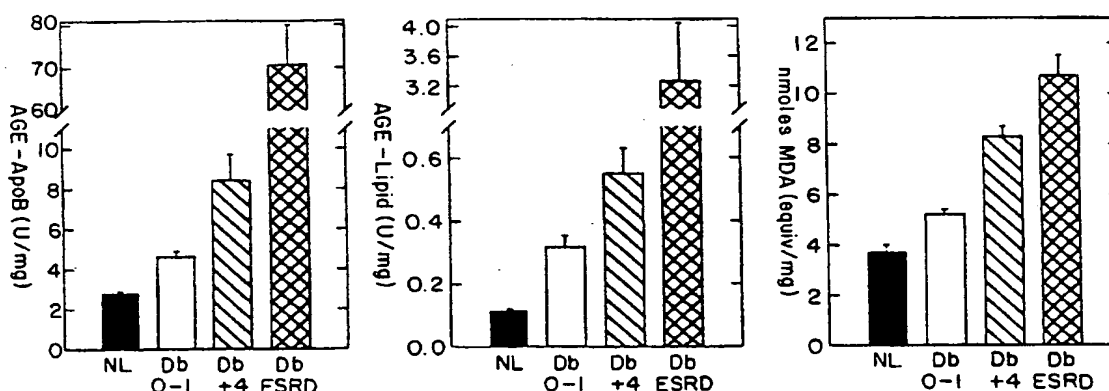
(57) Abstract

The *in vivo* oxidation of lipids and lipid-containing molecules has been discovered to be initiated by the concurrent reaction of such lipid materials with reducing sugars such as glucose, advanced glycosylation endproducts such as AGE-peptides, or a compound which forms advanced glycosylation endproducts, to form materials or particles known as AGE-lipids. AGE-lipids have been implicated in the aging process, the abnormal formation of lipofuscin and in various disease states such as diabetes and atherosclerosis. Diagnostic methods are contemplated, extending in utility from the detection of the onset and course of conditions in which variations in lipid oxidation, AGE-lipid levels, LDL levels, apolipoprotein levels, apolipoprotein receptor binding the like, may be measured, to drug discovery assays. Corresponding methods of treatment and pharmaceutical compositions are disclosed that are based on an active ingredient or ingredients that demonstrate the ability to modulate the levels of all the foregoing markers of lipid oxidation.

GLYCOSYLATION OF LIPIDS AND LIPID-CONTAINING PARTICLES, AND DIAGNOSTIC AND THERAPEUTIC METHODS AND MATERIALS DERIVED THEREFROM

5   This invention was made with partial assistance from
grant Nos. AGO-9453, AGO-6943 and DK 19655-15 from the
National Institutes of Health. The government may have
certain rights in this invention.

10   RELATED PUBLICATIONS

The Applicants are co-authors of the following articles
directed to the subject matter of the present invention:
Bucala, R., et al., "Glucose Reacts With Phospholipids to
15   Initiate Advanced Glycosylation and Fatty Acid Oxidation:
Mechanism for the Oxidative Modification of LDL *In Vivo*,
(submitted).

                    TECHNICAL FIELD OF THE INVENTION
20
The present invention relates generally to the non-
enzymatic glycosylation of proteins and other
biomolecules and the often consequent formation of
advanced glycosylation endproducts (AGEs), and
25   particularly to the formation of lipid-AGEs and the role
that glycosylated lipids and lipoproteins may play as
markers and actors in conditions such as atherosclerosis
and diabetes.

30   BACKGROUND OF THE INVENTION

Glucose and other reducing sugars attach non-
enzymatically to the amino groups of proteins in a
concentration-dependent manner. Over time, these initial
35   Amadori adducts undergo further rearrangements,
dehydrations and cross-linking with other proteins to
accumulate as a family of complex structures which are
referred to as Advanced Glycosylation Endproducts (AGEs).

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Beginning with the early work of the present applicants
and extending to the present, substantial progress has
been made toward the elucidation of the role and clinical
significance of advanced glycosylation endproducts, so
5   that it is now acknowledged that many of the conditions
heretofore attributed to the aging process or to the
pathological effects of diseases such as diabetes, are
attributable at least in part to the formation of AGEs *in
vivo*.

10

Advanced glycosylation tends to occur on molecules with
long half-lives, under conditions of relatively high
sugar concentration, such as in diabetes mellitus.
Numerous studies have suggested that AGEs play an
15   important role in the structural and functional
alteration which occurs during aging and in chronic
disease. Additionally, advanced glycosylation
endproducts are noted to form more rapidly in diabetic
and other diseased tissue than in normal tissue.

20

A particular area that has received attention in light of
the series of discoveries regarding the relationship of
advanced glycosylation of proteins to the etiology of
conditions such as diabetes and aging, has been the set
25   of events that coincide in the development of vascular
disease. Specifically, the formation of atherosclerotic
lesions and plaques is an example of a condition that has
been extensively investigated with a view to elucidating
the interrelationship, if any, that exists between the
30   oxidation of low density lipoproteins (LDL) and the
presence and formation of AGEs.

Oxidation of the lipid component of low-density
lipoprotein (LDL) results in the loss of the recognition
35   of the apo B component by cellular LDL receptors, and in
the preferential uptake of oxidized-LDL(ox-LDL) by
macrophage "scavenger" receptors. The enhanced

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endocytosis of ox-LDL by vascular wall macrophages
transforms them into lipid-laden foam cells that
characterize early atherosclerotic lesions.

5   Previous studies have suggested that AGE modification of
     LDLs increases the potential for lipid oxidation. The
     "family" of AGEs includes species which can be isolated
     and characterized by chemical structure; some being quite
     stable, while others are unstable or reactive. AGE-
10   lipids may also be stable, unstable or reactive.

     When used with reference to endogenous lipids, AGE-lipid
     compounds are typically formed non-enzymatically *in vivo*.
     However, AGE-lipid compounds can also be produced *in
15   vitro* by, e.g., incubating a mixture of a reducing sugar
     and a suitable lipid, e.g., a lipid bearing an amino
     group, or by other methods *in vitro*, such as chemical
     coupling of AGEs and AGE models to biological
     macromolecules.

20
     The reaction between reducing sugars and the reactive
     groups of lipids may initiate the advanced glycosylation
     process. This process typically begins with a reversible
     reaction between the reducing sugar and the reactive
25   group to form a Schiff base, which proceeds to form a
     covalently-bonded Amadori rearrangement product. Once
     formed, the Amadori product undergoes further
     rearrangement to produce the AGE-modified compound.

30   Although these reactions occur slowly, lipids may
     accumulate a measurable amount of AGEs *in vivo*. The
     resulting AGE-lipids may reduce the structural and/or
     functional integrity of organs and organ parts, modify
     the metabolism, or otherwise reduce or impair host
35   function.

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Picard et al. (1992) *Proc. Natl. Acad. Sci. USA*, 89:6876-
6880, studied the reaction between malonyl dialdehyde
(MDA) and apolipoprotein B (apo B), a protein component
of LDL, and performed experiments to determine the
5   ability of aminoguanidine to bind preferentially to MDA
to prevent its conjugation to apo B. To establish the
environment for these experiments, the authors induced
lipid peroxidation by incubation with endothelial cells
or with Cu^{2+}. However, the Picard et al. experiments are
10   limited by the specific *in vitro* environment chosen, as
the physiological oxidation of lipids to form the
reactive aldehydes to which aminoguanidine is confirmed
to bind will not occur by the means utilized in the
article.
15

More particularly, *in vitro* studies suggest that the
oxidative modification of lipids proceeds via free
radical-mediated oxidation of unsaturated bonds that are
present within fatty acid residues (12, 13).
20   Polyunsaturated fatty acids are particularly sensitive to
oxidation because methylene hydrogens located between
paired double bonds are easily abstracted by radical-
catalyzed reactions. Diene conjugation occurs and
hydroperoxides form. This is followed by fatty acid
25   decomposition, the formation of reactive aldehydes, and
in the case of LDL, the covalent modification of
apoprotein residues (12, 14, 15).

The biochemical processes that initiate lipid oxidation
30   *in vivo* remain poorly understood. Triplet oxygen is a
poor oxidant under normal, physiological conditions and
significant oxidation of LDL *in vitro* occurs only after
the addition of micromolar concentrations of divalent
metals such as copper. Lipid oxidation is prevented
35   completely in these incubations by the inclusion of metal
chelators such as EDTA (15). LDL oxidation also occurs
'in diverse cell culture systems and can be inhibited

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partially by pharmacological blockade of cellular
lipoxygenases (16). The precise role of reactive oxygen
species in the oxidative modification of lipids *in vivo*
has not been determined, however. Low trace metal
5   concentrations, the high availability of ligands that
form tight coordination complexes with metals, and the
abundant anti-oxidant capacity of plasma suggest that
metal-catalyzed autoxidation and reactive oxygen species
play little, if any role in mediating lipid oxidation *in*
10   *vivo* (17-19).

Further studies disclosed herein have more fully revealed
and likewise confirmed the significance of the findings
presented therein. Accordingly, it is toward the
15   presentation of these findings and the further
elaboration of the various earlier stated embodiments of
the invention that the present disclosure is directed.

SUMMARY OF THE INVENTION

20

In a first aspect of the invention, the *in vivo* oxidation
of lipids has been determined to be initiated by the
reaction of such lipids to form AGE-lipids as defined
herein. Accordingly, the invention extends to a method
25   for modulating the *in vivo* oxidation of lipids by
controlling the formation and presence of AGE-lipids. A
corresponding diagnostic utility comprises the
measurement of the course and extent of *in vivo* lipid
oxidation by a measurement of the presence and amount of
30   AGEs and particularly, AGE-lipids as defined herein. An
assay is included that may use the AGE-lipids of the
present invention to identify disease states
characterized by the presence of AGE-lipids.
Additionally, such an assay can be utilized to monitor
35   therapy and thus adjust a dosage regimen for a given
disease state characterized by the presence of AGE-
lipids.

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More particularly, as the *in vivo* oxidation of lipids is
related to the onset and course of atherosclerosis, the
control of *in vivo* lipid oxidation represents a

5   therapeutic strategy for its treatment, and the invention
thus comprises a method for treating atherosclerosis by
inhibiting the formation of AGE-lipids. Likewise, the
measurement of AGE-lipid levels in mammals represents a
method for diagnosing the likelihood or onset of

10   atherosclerosis, or measuring the course or severity of
the disease.

As noted above, AGE-lipids are useful as markers of a
variety of conditions in which the fluctuation in lipid

15   levels may reflect the presence or onset of dysfunction
or pathology. AGE-lipids are also lipid-soluble and are
useful alone and in conjunction with known carriers and
delivery vehicles, such as liposomes, for the transport
of therapeutic and other agents, including in certain

20   instances the AGE moieties themselves, across membranes
and epithelial layers, for example, to particular sites
in a patient for treatment. The particular site of
interest may be one which has at least one AGE receptor
which recognizes the AGE-lipid or a portion thereof.

25
A method of preparing AGE-lipids is also disclosed which
comprises incubating the lipid with an advanced
glycosylation endproduct or a compound which forms
advanced glycosylation endproducts for a length of time

30   sufficient to form said AGE-lipid.

Pharmaceutical compositions are also disclosed that
comprise an AGE-lipid in combination with a
pharmaceutically acceptable carrier. Such pharmaceutical

35   compositions may include an additional active agent(s) in
some instances, and may be prepared and used for oral,
parenteral or topical, e.g., transdermal, sublingual,

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buccal or transmucosal delivery. As stated, the
pharmaceutical compositions can be in the form of a
liposome in certain instances.

5   Further, AGE-lipids also demonstrate therapeutic utility
and may accordingly be prepared as described above, for
administration in controlled quantities to stimulate the
uptake and removal of senescent macromolecules, to
promote skin rejuvenation or remodeling by such activity,
10 and to serve as a drug delivery means. In this
connection, the AGE-lipids and pharmaceutical
compositions containing them may be prepared and
administered as and where appropriate.

15 A further embodiment of the invention relates to the
concomitant discovery that the *in vivo* oxidation of LDL
is likewise initiated by the formation of LDL advanced
glycosylation endproducts (AGE-LDL). AGE-LDL may be
formed by reaction with glucose or another *in vivo*-
20 resident reducing sugar, an advanced glycosylation
endproduct, or active fragments thereof, including AGE
peptides circulating in the serum of a mammal. More
particularly, the formation of AGE-LDL comprises the
attachment of AGE moieties to either or both the lipid
25 and apoprotein components, in the latter instance to form
AGE-apo B.

Apo B, in turn, has a region within its receptor binding
domain that is susceptible to AGE modification. This
30 site may be protected from AGE modification as part of a
therapeutic strategy, and may also serve as the focal
point of a drug discovery assay or receptor assay in a
diagnostic context.

35 Accordingly, the invention includes a method for
diagnosing or monitoring conditions in which serum LDL or
cholesterol levels are abnormal comprising measuring the

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presence and amount of a marker selected from AGE-lipids, AGE-LDL and AGE-apo B. The stated method may be used for example, to diagnose or monitor atherosclerosis and diabetes. A corresponding therapeutic method comprises

5 the treatment of a mammal to modulate, and in the majority of instances, to lower serum LDL or cholesterol levels, by the administration of an agent that serves to modulate AGE-LDL levels, and specifically to inhibit the formation of AGE-LDL.

10

Also, a method of modulating lipid metabolism in a mammal in need of such treatment is included. The method comprises administering to said mammal a lipid metabolism-modulating effective amount of an agent that

15 can modify the recognition and removal of lipids from serum, and more particularly, such agents as can modify the recognition and binding of apo B by LDL receptors.

Generally, the therapeutic methods of the present

20 invention contemplate the inhibition of *in vivo* lipid oxidation, LDL level increases or apo B modifications, by the administration of an agent or a pharmaceutical composition containing such agent or a plurality of such agents, for the inhibition of the formation of advanced

25 glycosylation endproducts involving any or all of the lipid and lipid-related materials subject to such *in vivo* oxidation. Such agents comprise antagonists of advanced glycosylation, and include antibodies to AGEs, antibodies to AGE-lipids, antibodies to AGE-LDL, antibodies to AGE-

30 apo B, as well as other ligands that would bind and neutralize the foregoing antigens. Suitable agents may also be selected from those agents that are reactive with an active carbonyl moiety on an early glycosylation product, and preferably are selected from aminoguanidine,

35 α-hydrazinohistidine, analogs of aminoguanidine, and pharmaceutical compositions containing any of the foregoing, all as recited in detail herein. The

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inventions set forth herein contemplate the discovery of
additional agents that may then be used in like fashion
and for like purpose.

5   In an alternate embodiment, the *in vivo* oxidation of
lipids, once initiated, is driven by the presence and
activity of the lipid peroxidation breakdown products.
These include lipid peroxides, as well as highly reactive
aldehydes such as malonyl dialdehyde (MDA). These
10   aldehydes can react with and/or crosslink to proteins,
for example, through available free amino groups.
Inhibitors of AGE formation such as aminoguanidine may be
used to inhibit the activity of these reactive aldehydes
by reacting directly with them. Accordingly, a
15   therapeutic strategy for the treatment of atherosclerosis
or other conditions in which LDL levels, cholesterol
levels or lipid levels generally, are undesirably high
comprises the administration of a therapeutically
effective amount of an agent capable of neutralizing the
20   activity of the reactive aldehyde products of *in vivo*
lipid oxidation. Preferred inhibitors include the agents
and antagonists recited above, and other materials
disclosed herein.

25   Accordingly, it is a principal object of the present
invention to modulate and control the *in vivo* oxidation
of lipids and lipid-like moieties by controlling the
formation of advanced glycosylation endproducts (AGEs),
and particularly AGEs involving such lipid and lipid-like
30   moieties.

It is a further object of the present invention to
provide a method for diagnosing conditions in which
abnormal lipid oxidation is a characteristic, by
35   detecting and measuring the presence and extent of lipid-
AGE formation.

10

It is a still further object of the present invention to
provide a method for diagnosing and treating
atherosclerosis by measuring and inhibiting the formation
of AGE-lipids.

5

It is a still further object of the present invention to
provide a method for lowering serum LDL levels, by
inhibiting the formation of AGEs including AGE-lipids.

10   It is a still further object of the present invention to
provide a method for identifying new drugs and
corresponding agents capable of treating abnormal lipid
oxidation, by use of an assay involving AGE-lipids.

15   It is yet another object is to utilize AGE-lipids to
treat certain diseases and conditions, such as skin
conditions, or to utilize the AGE-lipid moieties for
purposes of delivering disease-treating medications to
particular biologically active sites.

20

It is a still further object of the present invention to
identify AGE-lipids and methods of inhibiting the
formation in instances or disease conditions where the
presence or biological activity of these AGE-lipids is
25   detrimental to the host organism, or indicative of the
presence of a disease state in the host organism.

Other objects and advantages will be apparent from a
consideration of the ensuing detailed description which
30   proceeds with reference to the following illustrative
drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

35   FIGURE 1 is a graph of absorbance at 360 nm of AGE-
compound formation over time using various lipids
incubated with glucose and/or aminoguanidine;

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FIGURE 2 is a graph of fluorescence intensity using
various lipids incubated with glucose and/or
aminoguanidine;

5   FIGURE 3 is a graph of lipid oxidation in relation to
AGE-lipid formation using various lipids incubated with
glucose and/or aminoguanidine;

FIGURE 4 is a graph of changes in ultraviolet and visible
10 absorbance spectra (200-500 nm) over time (0-50 days) of
phosphotidylethanolamine (PE) incubated with 500 mM
glucose;

FIGURE 5 is a graph of AGE-specific absorbance as a
15 function of glucose concentration;

FIGURE 6 is a graph of AGE-specific fluorescence as a
function of glucose concentration;

20 FIGURE 7 is a graph of lipid oxidation changes as a
function of glucose concentration;

FIGURE 8A is a graph showing the concentration-dependent
reaction between malonyldialdehyde (bis-[diethylacetal])
25 (MDA) (0-20 μM) and aminoguanidine (0-500 μm);

FIGURE 8B is a graph of the inhibition of
thiobarbiturate activity as a function of AG
concentration.

30
FIGURES 9A-C are graphs of the comparison measurement of
AGE and oxidative modification of human LDL isolated from
plasma of 8 normoglycemic, non-diabetic individuals (o)
and 16 patients with diabetic mellitus: FIGURE 9A
35 compares the AGE modification of LDL apoprotein of non-
diabetic and diabetic patients. FIGURE 9B compares the
AGE modification of LDL lipid of non-diabetic and

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diabetic patients; and FIGURE 9C compares the oxidative
modification of LDL of non-diabetic and diabetic
patients. Values shown are the mean of duplicate
determinations.

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FIGURES 10A-C are graphs of time-dependent reaction of
human LDL (2.5 mg/ml) with glucose (200 mM). At time
intervals, samples were dialyzed against PBS/EDTA and
portions separated into lipid (FIGURE 10B) and apoprotein
10   (FIGURE 10A) components for AGE determination or assayed
for the presence of ox-LDL (FIGURE 10C). LDL incubated
with 200 mM glucose (Δ). LDL incubated with 200 mM
glucose and 300 mM aminoguanidine (▲). LDL incubated
alone (o). LDL incubated with aminoguanidine (•).
15   Values shown are the mean of duplicate determinations.

FIGURE 11A presents histograms depicting the accumulation
of AGEs on apo B that was isolated after incubation of
LDL with AGE-peptides, with or without co-incubation with
20   aminoguanidine, as detected in an AGE-specific ELISA.

FIGURE 11B presents histograms depicting the accumulation
of AGEs in a lipid fraction that was isolated after
incubation of LDL with AGE-peptides, with or without co-
25   incubation with aminoguanidine, as detected in an AGE-
specific ELISA.

FIGURE 12A presents histograms depicting the accumulation
of AGEs (as detected in an AGE-specific ELISA) on apo B
30   that was isolated from serum LDL obtained from human
study subjects who were non-diabetic (normal) or diabetic
with different numbers of diabetic complications, and
diabetic patients with end stage renal disease (ESRD).

35   FIGURE 12B presents histograms depicting the accumulation
of AGEs (as detected in an AGE-specific ELISA) in a lipid
fraction that was isolated from LDL obtained from human

study subjects who were non-diabetic (normal) or diabetic
with different numbers of diabetic complications, and
diabetic patients with end stage renal disease (ESRD).

5   FIGURE 12C presents histograms depicting the accumulation
of oxidized LDL (detected as MDA equivalents) in plasma
LDL that was obtained from human study subjects who were
non-diabetic (normal) or diabetic with different numbers
of diabetic complications, and diabetic patients with end

10 stage renal disease (ESRD).

FIGURE 13 presents histograms depicting the accumulation
of AGEs (as detected in an AGE-specific ELISA) on apo B
that was isolated from plasma LDL obtained from human

15 study subjects who were non-diabetic (normal) or diabetic
and with or without end stage renal disease.

FIGURE 14 presents histograms depicting the relative
lowering of LDL levels (shown as percent of pretreatment

20 baseline) in diabetic patients at the end of a 28-day
trial of aminoguanidine versus placebo.

DETAILED DESCRIPTION OF THE INVENTION

25 Numerous abbreviations are used herein to simplify the
terminology used, and to facilitate a better
understanding of the invention. The following
abbreviations are representative.

30 As used herein, the term "AGE-" refers to the compound
which it modifies as the reaction product of either an
advanced glycosylation endproduct or a compound which
forms AGEs and the compound so modified, such as the
lipid moiety. AGE-lipids can be formed *in vitro* by

35 reacting a lipid as defined herein with an AGE, such as
AGE-peptide, or either *in vitro* or *in vivo* with a

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compound such as a reducing sugar, e.g., glucose, until the lipid is modified to form the AGE-lipid.

"Lipid" is used in the conventional sense to refer to
5   materials that are soluble to a greater or lesser degree in organic solvents, like alcohols, and relatively insoluble in aqueous media. Thus, the term "lipid" includes compounds of varying chain length, from as short as about 2 carbon atoms to as long as about 28 carbon
10   atoms. Additionally, the compounds may be saturated or unsaturated, and in the form of straight- or branched-chains or in the form of unfused or fused ring structures. Further, these lipid compounds can be optionally linked to other moieties, so long as at least
15   one primary amino group, or other crosslinkable or otherwise reactive group, is present in the molecule.

The term "lipid-related materials" is used herein to encompass not only lipids as conventionally understood
20   and as defined above, but those particles, aggregates and components thereof that are found in connection with lipid moieties. Examples of lipid-related materials included herein include fatty acids, sterol-type molecules, triglycerides, phospholipids, and lipoproteins
25   including apolipoproteins. Preferred lipid-related materials include as the AGE-reactive groups one or more primary amino groups. It is particularly preferred to include at least one primary amino group reactive with AGEs and compounds which form AGEs.
30
The lipid-related materials that are of primary interest are those that react to form advanced glycosylation endproducts. The resulting AGEs are given herein the common designation of "AGE-lipid(s)" for purpose of
.35 convenience and consistency, it being understood that this designation will include within its scope AGEs formed literally with lipid moieties alone, as well as

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AGEs formed with lipid-related materials such as apolipoproteins. The use of the term "AGE-lipid(s)" in accordance with the present invention is therefore intended to cover such diverse materials within its

5   scope.

Those lipid-related materials that are preferably used in the preparation of the AGE-lipids affirmatively used in the diagnostic and therapeutic methods of the present

10   invention, are phospholipid compounds containing primary amino groups, such as phosphatidylethanolamine. Other lipid-related materials also useful in the present invention are the lipoproteins, particularly those involved in atherogenesis, i.e., low-density lipoproteins

15   (LDLs), and the apolipoproteins that comprise the protein component of LDL, and in particular, apolipoprotein B (apo B).

The AGEs that may be employed to prepare AGE-lipids

20   include such species as 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole ("FFI"); 5-hydroxymethyl-1-alkylpyrrole-2-carbaldehyde ("Pyrraline"); 1-alkyl-2-formyl-3,4-diglycosyl pyrrole ("AFGP"), a non-fluorescent model AGE; carboxymethyllysine; and pentosidine. These compounds

25   have been isolated and characterized as the reaction products which form following the formation of Amadori reactions. However, the *in vivo* formation of AGEs and the incubation of lipids with AGEs or compounds which form AGEs likely forms AGE species not recited above.

30   Consequently, the invention is not limited to these precise chemical compounds, since other AGE compounds can be formed or have a role in accordance with the teachings herein. Thus, the term AGE can refer to the advanced glycosylation endproduct which the lipid is reacted with

35   as well as the particular form which is produced according to the reaction.

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As stated earlier, the present invention is related in part to the discovery that a relationship exists between lipid oxidation and metabolism, and the *in vivo* formation of advanced glycosylation endproducts on lipid-related
5   materials as defined hereinabove (AGE-lipids).
Particularly, and as supported by the data presented herein, AGE-lipids appear to initiate lipid oxidation, reduce the efficiency and operation of the body mechanism for LDL clearance by the interruption of normal receptor
10 binding between the apolipoprotein B portion of LDL and appropriate LDL receptors, and leads to increased levels of plasma lipids such as low density lipoproteins (LDL).

This latter observation is believed to reflect the
15 existence of a site for the formation of AGEs that is either adjacent or within the LDL receptor binding domain of apo B. The result of the formation of an AGE on apo B is believed to subject apo B to preferential uptake by macrophage "scavenger" receptors with concomitant
20 inhibition of interaction with the LDL receptor, with the result that the LDL molecules are avidly consumed by the macrophage leading to the formation of undesired foam cells that contribute to atherosclerotic plaque formation. It is therefore of primary interest and
25 importance as a therapeutic strategy, to treat atherosclerosis in more than one way, first by the inhibition of AGE formation and lipid oxidation that, in turn, leads to increased plasma LDL levels, and secondly, to restore the full functionality of apo B uptake by LDL
30 receptors and to avert the formation of undesired foam cells by the consumption of LDL by the macrophage.

A further discovery in accordance with the present invention that forms yet an additional aspect thereof, is
35 the observation that the oxidation of lipids, while initiated by AGE-lipid formation, is further perpetuated by the circulation of certain lipid oxidation byproducts.

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Also, fatty acid oxidation products such as the malonyl
dialdehyde-like compounds participate in protein
modification by reaction with free available amino
groups. It therefore is desirable as a further
5   therapeutic strategy to neutralize the activity of these
oxidation byproducts by a reaction of an appropriate
neutralizing agent therewith.

As part of the present invention, the inhibitors of
10 advanced glycosylation identified earlier and listed
herein in detail, including aminoguanidine, α-
hydrazinohistidine, lysine and corresponding analogs,
have been found and confirmed to react in such fashion
with the MDA-like byproducts that are produced in vivo
15 and to neutralize the same so that they no longer
participate in the modification of proteins. The present
invention is not considered to be limited or to depend
upon a particular mechanism of action, and the foregoing
is merely illustrative of the observed beneficial
20 activity of the noted inhibitors.

In view of the above, the present invention includes a
dual therapeutic strategy where agents such as
aminoguanidine may be administered to inhibit in vivo
25 AGE-lipid formation and consequent initiation of lipid
oxidation, and to react with any byproducts of an ongoing
lipid oxidation to prevent reaction of these byproducts
with proteins as described above.

30 More particularly, the present invention relates to a
method of modulating lipid metabolism including the
control and adjustment of such metabolism either to
increase or decrease same, by the administration to a
mammal or host in need of such treatment, of a lipid
35 metabolism-modulating effective amount of a particular
agent or group of agents that are capable of modifying
the recognition and removal of lipids from serum, which

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agents are importantly capable of controlling the
formation of AGEs, and particularly AGE-lipids. In
selected instances, such as where predetermined
quantities of AGE-lipids may act to stimulate the systems
5 of the host to adjust lipid metabolism for therapeutic
benefit, the method includes the administration of such
AGE-lipids by means described herein in detail.

The lipids subject to advanced glycosylation are as
10 recited earlier, selected from amine-containing lipids,
low-density lipoproteins, and apolipoproteins, and
particularly in the last mentioned instance, apo B. The
method may be practiced to lower low-density lipoprotein
levels in a patient, and is applicable for example, to
15 the prevention and/or treatment of hypercholesterolemia,
atherosclerosis, and kidney failure.

The agents contemplated for use in this method include
materials selected from the group consisting of
20 antibodies against advanced glycosylation endproducts,
ligands, including AGE receptors and active fragments
thereof, capable of binding to and neutralizing advanced
glycosylation endproducts, and compounds capable of
inhibiting the formation of advanced glycosylation
25 endproducts. Suitable antibodies include polyclonal
antibodies, monoclonal antibodies, chimeric antibodies,
and active fragments thereof, all as discussed in detail
below. These agents are administered to restore
effective lipid metabolism and to correspondingly reduce
30 lipid oxidation. The foregoing appreciates that lipid
metabolism is in part controlled by the effective binding
of the apolipoprotein apo B to the LDL receptor, so that
any compounds or agents contemplated for use in this
aspect of the invention, would be capable of interacting
35 with the receptor binding domain of apo B to avert the
formation of AGEs adjacent to or therewithin, and to

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render such receptor binding domain recognizable by the
LDL receptor.

As mentioned previously and as supported by one of the
5 examples presented later on herein, it has been observed
that such AGE-peptides take an affirmative role in the
formation of AGE-lipids and likewise participate in the
promotion and acceleration of lipid oxidation and the
various consequences thereof. It is therefore desirable,
10 in the instance where such activity is to be inhibited,
to neutralize such AGE-peptides by reacting with them to
prevent them from promoting further AGE formation. In
this connection, aminoguanidine and like inhibitor
compounds can be administered.
15
    Concomitant with the above therapeutic strategies are
effective diagnostic protocols that may be employed to
determine the onset and course of a condition whose
measurable variable may include lipid oxidation, by
20 resort to the detection and measurement of the extent of
advanced glycosylation of lipids. More particularly,
AGE-lipid formation may be detected by means such as the
AGE-ELISA developed by the present inventors, to
determine the extent of AGE-lipid formation and to
25 thereby assess the extent of lipid oxidation, and
consequent effects on plasma LDL levels. Accordingly,
measurable increases in lipid oxidation and plasma LDL
levels will signal the development and onset of
hypercholesterolemia and atherogenesis, so that the
30 present method may be effectively employed to diagnose
and monitor the development of vascular disease, and
particularly atherosclerosis. Likewise, the presence of
AGE-lipids is also reflective of the development and
existence of diabetic conditions such as diabetic
35 retinopathy, diabetic and non-diabetic nephropathy, and
the like, so that the present diagnostic methods may be

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used to measure the development and severity of these
conditions as well.

With respect to the effect that advanced glycosylation
5   endproduct formation exerts on the ability to clear low
density lipoproteins by the recognition and binding of
apo B to the LDL receptor, the present invention
contemplates and includes the full identification of the
receptor binding domain of apo B, and particularly those
10 portions of the receptor binding domain that are
presently susceptible to AGE formation. For example, and
as set forth later on herein, Applicants have discovered
that a particular segment of the receptor binding domain
defines lysine with flanking arginine residues, that most
15 likely serves as a site for the formation of the advanced
glycosylation endproduct with apo B.

Accordingly, the receptor binding domain may serve as the
focal point for a drug discovery assay, where, for
20 example, apo B may be immobilized, and incubated both
with agents conducive to the formation of an AGE on the
binding domain thereof, and a quantity of a particular
drug or inhibitory agent under test. The extent to which
the drug serves to either bind with the AGE and thereby
25 inhibit apo B AGE formation, or binds directly with apo B
and thereby prevents the same, could then be measured.
This particular assay could be prepared as a receptor
assay in conjunction with the LDL receptor, to determine
whether the apo B receptor binding domain is disabled
30 after incubation with an AGE/AGE-forming materials and a
particular drug under test. Both possibilities for drug
discovery assays are contemplated herein and are
considered within the scope of the present invention.

35 Both the diagnostic and therapeutic methods of the
present invention contemplate the use of agents that have
an impact on the formation of AGE-lipids. Among these

agents, antibodies to AGEs and other ligands may be
prepared and used. These terms are defined below.

The term "antibody" includes any immunoglobulin,
5   including antibodies and fragments thereof that binds a
specific epitope, and such general definition is intended
to apply herein. The term therefore encompasses
polyclonal, monoclonal and chimeric antibodies, the last
mentioned described in further detail in U.S. Patent Nos.
10   4,816,397 and 4,816,567.

Also, an "antibody combining site" is that structural
portion of an antibody molecule comprised of heavy and
light chain variable and hypervariable regions that
15   specifically bind antigen. Exemplary antibodies include
antibody molecules such as intact immunoglobulin
molecules, substantially intact immunoglobulin molecules
and those portions of an immunoglobulin molecule that
contain the active binding site, including those portions
20   known in the art as Fab, Fab', $F(ab')_2$ and F(v), which
portions are preferred for use in therapeutic methods
associated herein.

Fab and $F(ab')_2$ portions of antibody molecules are
25   prepared by the proteolytic reaction of papain and
pepsin, respectively, on substantially intact antibody
molecules by methods that are well-known. See for
example, U.S. Patent No. 4,342,566 to Theofilopolous et
al. (The disclosures of the art cited herein are hereby
30   incorporated by reference.) Fab' antibody molecule
portions are also well-known and are produced from $F(ab')_2$
portions followed by reduction of the disulfide bonds
linking the two heavy chain portions as with
mercaptoethanol, and followed by alkylation of the
35   resulting protein mercaptan with a reagent such as
iodoacetamide.

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The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal

5 antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. An antibody may be prepared having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) antibody.

10

Likewise, the term "ligands" includes such materials as AGE derivatives that would bind to AGE-binding partners, and would include such materials as are prepared by the reaction of AGEs with avidin or biotin, or the

15 preparation of synthetic AGE derivatives that may be prepared from reducing sugars such as glucose, glucose-6-phosphate (G-6-P), fructose and ribose, and peptides, proteins and other biochemicals such as bovine serum albumin (BSA), avidin, biotin derivatives, and enzymes

20 such as alkaline phosphatase. Likewise, enzymes and other carriers that have undergone advanced glycosylation may also serve as ligands in any of the assays of the present invention. Accordingly, carriers such as carbohydrates, proteins, synthetic polypeptides, lipids

25 and biocompatible natural and synthetic resins, and any mixtures of the same may be reacted with sugars to form advanced glycosylation endproducts and may thereby be useful in the present methods. The present diagnostic methods are intended to contemplate all of the foregoing

30 materials within their scope.

The term "AGE binding partners" is intended to extend to anti-AGE antibodies and to other cellular AGE binding proteins or receptors for AGEs, which AGEs may be found

35 on peptides, molecules and cells.

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As discussed above, the present invention extends to the
preparation and use of AGE-lipids in a variety of
diagnostic and therapeutic contexts. With respect to the
formation of AGE-lipids, the compounds which form AGEs
5   are typically reducing sugars. Reducing sugars or the
AGEs themselves can react with the lipids to form AGE-
lipids. However, in *in vitro* techniques, it is likely
that a compound which forms AGEs, such as a reducing
sugar, will be used. Examples of reducing sugars include
10   glucose, fructose, ribose and glucose-6-phosphate.

AGE-lipids can be affirmatively used in the treatment,
rejuvenation or remodeling of skin. For example, the
AGE-lipids can be administered in an amount effective for
15   treating skin ailments or rejuvenating or remodeling the
skin, such as to remove or induce the removal of
wrinkles. By way of explanation, but not limitation, it
is postulated that the application of AGE-lipids to the
skin may attract cells, e.g., macrophages, which have the
20   ability to remove naturally occurring AGE-compounds
generally from the site of deposition. As a result, *in
vivo* generated and naturally deposited AGE-compounds may
be removed and also AGE-lipids and other AGEs may induce
cells, e.g., macrophage, T-cells and endothelial cells
25   and fibroblasts, to secrete a variety of substances,
e.g., cytokines, growth factors and effector molecules,
such as TNF, IL-1, IGF-1, PDGF and other compounds, and
collagenase and thereby modulate biological processes,
e.g. skin remodeling and wound healing.
30
The AGE-lipids can be applied to the skin in the form of
topical preparations for cosmetic or medicinal use in the
form of, e.g., creams, gels or ointments, or can be
incorporated into pharmaceutical preparations with other
35   ingredients. Likewise, the topical use of the AGE-lipid
compounds described herein could include oth r agents

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useful for the treatment of skin ailments or disease,
e.g., wrinkling, acne, wound healing, etc.

The AGE-lipids of the present invention can also be
5   applied to the skin to modify the effect or use of other
medicinal agents. For example, the AGE-lipids could be
applied to the skin in conjunction with anti-inflammatory
or anti-infective therapeutic agents or other compounds
which are effective topically or transdermally.
10   Likewise, such AGE-lipids may enhance the penetration or
activity of the other compounds administered in
combination.

Additionally, the AGE-lipids may function to attract
15   cells or other endogenous components, e.g., antibodies,
which are effective in removing AGEs from the system, or
which function in the removal of such compounds from the
system. By providing AGE-lipids to the desired site,
these cells and other components may be attracted to the
20   area of application and induced to remove other harmful
components.

Another aspect of the present invention relates to
compositions which can be in any pharmaceutically
25   acceptable form, e.g., transdermal, oral, parenteral,
topical (via the skin, inhalation, transmucosally, e.g.,
rectally, vaginally, buccally or sublingually) as well as
other dosage forms administered by other routes of
administration. Such compositions typically contain an
30   AGE-lipid which is effective for treating the particular
disease or condition, or is effective for attracting,
activating or inducing the activity of cells or
antibodies to the area of interest in an effort to
control, reduce or eliminate the formation of lipofuscin,
35   and other amyloid materials. The amount of the AGE-lipid
present in the composition and thus the amount
administered will depend upon the particular condition

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under treatment, as well as the age, weight, and
condition of the patient.

Also, the AGE-lipids of the present invention may be
5   useful for the enhancement of the activity of other drugs
or therapeutic agents. For example, the AGE-lipid can be
coadministered or administered separately from another
drug to take advantage of the lipid solubility of the
preferred AGE-lipids which are useful herein. Likewise,
10   the drugs can be used essentially simultaneously to
attract cells or other biological components, e.g.,
antibodies, which are to be treated or are necessary or
desired in the pharmacological site of activity for
purposes of enhancing the activity of the AGE-lipid
15   and/or the other drug.

Another aspect of the invention relates to pharmaceutical
dosage forms such as liposomes. Liposomes can be used
with AGE-lipids present on the outer layer thereof, or
20   incorporated into the interior, based upon the lipid
solubility of the AGE-lipid, the relative size of the
liposomes, the presence of other therapeutic agents
contained therein, the mode and biological site of
intended use/activity and numerous other factors.

25
Preferred AGE-lipids for use as described herein may take
advantage of cellular AGE binding proteins or AGE-
receptors as well as the other physical parameters of the
AGE-lipids described in the present invention. By way of
30   non-limiting example, the synthetic and naturally
occurring AGE, FFI, is recognized by and reactive with
macrophage cells (macrophage cells have AGE receptors
which recognize FFI) but not particularly reactive with
endothelial cells. Thus, if a liposome or other
35   pharmaceutical dosage form containing AGE-lipids is to be
delivered such that macrophage cells are targeted, the
FFI moiety can be included. In this manner, differences

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in AGE receptor activity and in the reactivity of
different AGEs can be taken advantage of.

   Likewise, the AGE-lipids of the present invention can be
5 used to produce antibodies to AGE-lipids, and these
   antibodies can be used as described herein. For example,
   antibody formation can be induced by injecting a mammal
   with an immunogen comprised of an AGE-lipid and then
   collecting the serum of the mammal. Such serum will
10 typically contain antibodies which recognize and bind to
   AGE-lipids. These antibodies may be polyclonal or
   essentially monoclonal, and may be prepared e.g., by
   using an appropriate immunization protocol, such as a
   hyperimmunization protocol. Accordingly, appropriate
15 fusion, plating, screening, selection and replication
   techniques can be utilized to obtain monoclonal
   antibodies which recognize specific epitopes on the
   particular AGE-lipid utilized.

20 The AGE-lipids, antibodies and compositions can also be
   used in the assessment of the quality, preservation or
   degradation of stored foods or other biological
   substances. For example, the presence and concentration
   of advanced glycosylation endproducts can be identified.
25 This technique is particularly useful in identifying
   undesirable concentrations of advanced glycosylation
   endproducts that accumulate with prolonged storage.

   The AGE-lipids, antibodies and compositions can be used
30 in the diagnosis and assessment of certain diseases. For
   example, the location and concentrations of advanced
   glycosylation endproducts in the body could be
   identified. This technique is particularly useful in
   identifying undesirable concentrations of advanced
35 glycosylation endproducts, such as atheromatous plaques,
   or for the identification of complications of disease
   states such as diabetes mellitus.

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Of particular diagnostic importance is the identification
of AGE-lipids wherein the lipid is a low-density
lipoprotein (LDL). In the case of LDL, incubation with
glucose or AGE-peptides produces AGE moieties that are
5   linked to both the lipid and to the apoprotein
components. Oxidized-LDL forms concurrently with AGEs
during these incubations. Aminoguanidine, as well as
other known agents for the inhibition of the advanced
glycosylation of proteins, inhibits both the advanced
10   glycosylation and oxidative modification processes.
Analysis of LDL specimens isolated from the plasma of
diabetic individuals reveals increased levels of AGEs on
both the apoprotein and lipid components when compared to
normal, non-diabetic individuals. The level of LDL
15   oxidation also correlates significantly with AGE
modification, indicating that advanced glycosylation may
play a primary role in the generation of oxidized lipid
in vivo, and that this activity is inhibited by
aminoguanidine.

20

Thus, the identification, by standard assay procedures,
of high levels of AGE-LDL in a patient can be utilized to
ascertain the precise disease state, as well as to
monitor the efficacy of a therapeutic regimen, such as by
25   treatment with an AGE-inhibitor. In a particularly
preferred embodiment, the levels of AGE-LDL in a patient
can be utilized to diagnose the onset, severity or risk
for the development of diabetic conditions and
complications in the patient. Additionally, the level of
30   AGE-LDL in a patient can be utilized to diagnose the
onset or severity of atherosclerosis and associated
conditions in a patient.

The method comprises an assay involving in addition to
35   the analyte, one or more binding partners of AGE-lipids
and one or more ligands.

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Accordingly, the present assay method broadly comprises
the steps of:

A.   preparing at least one biological sample
suspected of containing said AGE-lipids;

5   B.   preparing at least one corresponding
binding partner directed to said sample;

C.   placing a detectable label on a material
selected from the group consisting of said sample, a
ligand to said binding partner and said binding partner;

10   D.   placing the labeled material from Step C
in contact with a material selected from the group
consisting of the material from Step C that is not
labeled; and

E.   examining the resulting sample for the
15 extent of binding of said labeled material to said
unlabeled material.

In a typical non-competitive assay in accordance with the
present invention, AGE-lipids are solubilized in methanol
20 and deposited on the assay plate by drying. The assay
plates are then hydrated and sequentially exposed to
anti-AGE primary antibodies and enzyme-conjugated second
antibodies specific for the primary antibodies, with
washing steps in between where appropriate. Enzyme
25 levels are then determined by, for instance, substrate
conversion protocols well known in the art, and the
amount of AGEs can thus be measured by reference to a
standard run in parallel.

30 In a typical competitive assay in accordance with the
present invention, an AGE binding protein or AGE receptor
may be combined with the analyte and a ligand, and the
binding activity of either or both the ligand or the
analyte to the receptor may then be measured to determine
35 the extent and presence of the advanced glycosylation
endproduct of interest. In this way, the differences in

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amounts bound between the components of the assay serves
to identify the presence and amount of the AGE-lipid.

The present invention also relates to a method for
5   detecting the presence of stimulated, spontaneous, or
idiopathic pathological states in mammals, by measuring
the corresponding presence of AGE-lipids. More
particularly, the activity of AGEs may be followed
directly by assay techniques such as those discussed
10   herein, through the use of an appropriately labeled
quantity of at least one of the binding partners to AGE-
lipids as set forth herein. Alternately, AGEs can be
used to raise binding partners or antagonists that could
in turn, be labeled and introduced into a medium to test
15   for the presence and amount of AGEs therein, and to
thereby assess the state of the host from which the
medium was drawn.

Thus, both AGE-lipids and any binding partners thereto
20   that may be prepared, are capable of use in connection
with various diagnostic techniques, including
immunoassays, such as a radioimmunoassay, using for
example, a receptor or other ligand to an AGE that may
either be unlabeled or if labeled, then by either
25   radioactive addition, reduction with sodium borotritiide,
or radioiodination.

In an immunoassay, a control quantity of a binding
partner to AGE-lipids may be prepared and optionally
30   labeled, such as with an enzyme, a compound that
fluoresces and/or a radioactive element, and may then be
introduced into a tissue or fluid sample of a mammal.
After the labeled material or its binding partner(s) has
had an opportunity to react with sites within the sample,
35   the resulting mass may be examined by known techniques,
which may vary with the nature of the label attached.

30

The labels most commonly employed for these studies are
radioactive elements, enzymes, chemicals which fluoresce
when exposed to ultraviolet light, and others.

5   Suitable examples of radioactive elements include ^3H, ^{14}C,
^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re.
In the instance where a radioactive label, such is
prepared with one of the above isotopes is used, known
currently available counting procedures may be utilized.
10

In the instance where the label is an enzyme, detection
may be accomplished by any of the presently utilized
colorimetric, spectrophotometric, fluorospectro-
photometric, thermometric, amperometric or gasometric
15   techniques known in the art. The enzyme may be
conjugated to the advanced glycosylation endproducts,
their binding partners or carrier molecules by reaction
with bridging molecules such as carbodiimides,
diisocyanates, glutaraldehyde and the like.
20

Many enzymes which can be used in these procedures are
known and can be utilized. The preferred are peroxidase,
ß-glucuronidase, ß-D-glucosidase, ß-D-galactosidase,
urease, glucose oxidase plus peroxidase, hexokinase plus
25   GPDase, RNAse, glucose oxidase plus alkaline phosphatase,
NAD oxidoreductase plus luciferase, phosphofructokinase
plus phosphoenol pyruvate carboxylase, aspartate
aminotransferase plus phosphoenol pyruvate decarboxylase,
and alkaline phosphatase. U.S. Patent Nos. 3,654,090;
30   3,850,752; and 4,016,043 are referred to by way of
example for their disclosure of alternative labeling
material and methods. A particular enzymatic detecting
material is anti-rabbit antibody prepared in goats and
conjugated with alkaline phosphatase through an
35   isothiocyanate.

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A number of fluorescent materials are known and can be
utilized as labels. These include, for example,
fluorescein, rhodamine and auramine. A particular
fluorescent detecting material is anti-rabbit antibody
5   prepared in goats and conjugated with fluorescein through
an isothiocyanate.

The AGE-lipids may be used to produce antibody(ies) to
themselves which can be produced and isolated by standard
10   methods including the well known hybridoma techniques.
The antibody(ies) can be used in another species as
through they were antigen(s) to raise antibody(ies).
Both types of antibody(ies) can be used to determine the
amount and location of the AGE-lipids in lipid masses,
15   whether in foodstuffs, or in the mammalian body. For
convenience, the antibody(ies) to the AGE-lipids will be
referred to herein as Ab_1 and antibody(ies) raised in
another species as Ab_2.

20   The degree of glycosylation in lipid masses suspected of
undergoing the same can be ascertained by the usual
immunological procedures applicable to such
determinations. A number of useful procedures are known.
Three such procedures which are especially useful utilize
25   either the AGE-lipid labeled with a detectable label,
antibody Ab_1 labeled with a detectable label, or antibody
Ab_2 labeled with a detectable label. The procedures may
be summarized by the following equations wherein the
asterisk indicates that the particle is labeled, and "Al"
30   stands for the AGE-lipid:

A. $Al*+Ab_1=Al*Ab_1$
B. $Al+Ab_1*=AlAb_1*$
C. $Al+Ab_1+Ab_2*=AlAb_1Ab_2*$

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The procedures and their application are all familiar to
those skilled in the art and accordingly may be utilized

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within the scope of the present invention. The
"competitive" procedure, Procedure A, is described in
U.S. Pat. Nos. 3,654,090 and 3,850,752. Procedure C, the
"sandwich" Procedure, is described in U.S. Patent Nos. RE
5   31,006 and 4,016,043. Still other procedures are known
such as the "Double antibody", or "DASP" procedure.

In each instance, the AGE-lipid substance forms complexes
with one or more antibody(ies) or binding partners and
10   one member of the complex is labeled with a detectable
label. The fact that a complex has formed and, if
desired, the amount thereof, can be determined by known
methods applicable to the detection of labels.

15   It will be seen from the above, that a characteristic
property of Ab_2 is that it will react with Ab_1. This is
because Ab_1 raised in one mammalian species has been used
in another species as an antigen to raise the antibody
Ab_2. For example, Ab_1 may be raised in rabbits and Ab_2 may
20   be raised in goats using Ab_1 as an antigen. Ab_2 therefore
would be anti-rabbit antibody raised in goats.

Accordingly, a test kit may be prepared for the
demonstration of AGE-lipids in a sample, whether in food
25   or in animals, comprising:
        (a) a predetermined amount of at least one labeled
immunochemically reactive component obtained by the
direct or indirect attachment of AGE-lipids or an AGE
binding partner to a detectable label;
30   (b) other reagents; and
        (c) directions for use of said kit.

More specifically, the diagnostic test kit may comprise:
        (a) a known amount of the AGE-lipid (or a binding
35   partner) generally bound to a solid phase to form a
immunosorbent, or in the alternative, bound to a suitable

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tag, or plural such components, etc. (or their binding
partners) one of each;

      (b) if necessary, other reagents; and

      (c) directions for use of said test kit.

5

In a further variation, the test kit may be prepared and
used for the purposes stated above, which operates
according to a predetermined protocol (e.g.
"competitive", "sandwich", "double antibody", etc.), and
10 comprises:

      a.   a labeled component which has been obtained by
coupling a AGE-lipid or a binding partner thereof to a
detectable label;

      b.   one or more additional immunochemical reagents
15 of which at least one reagent is a binding partner or an
immobilized binding partner, which binding partner is
selected from the group consisting of:

      (i) a binding partner capable of binding with the
labeled component (a);

20   (ii) a binding partner capable of binding with a
binding partner of the labeled component (a);

      (iii) a binding partner capable of binding with at
least one of the component(s) to be determined; and

      (iv) a binding partner capable of binding with at
25 least one of the binding partners of at least one of the
component(s) to be determined; and

      (c) directions for the performance of a protocol for
the detection and/or determination of one or more
components of an immunochemical reaction between the AGE-
30 lipid and a specific binding partner thereto.

By example, a solid phase assay system or kit may
comprise the solid substrate with either bound binding
partner and labeled AGE-lipid or bound AGE-lipid and
35 labeled binding partner. A sample to be assayed is then
placed in contact with the bound and unbound reagent and
a competitive reaction between the labeled material and

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any unlabeled binding partner(s) in the sample will cause
the retention of a dependent quantity of the former on
the solid substrate, whereupon it can be precisely
quantitatively identified. The foregoing explanation of
5   a particular competitive assay system is presented herein
for purposes of illustration only, in fulfillment of the
duty to present an enabling disclosure of the invention.
It is to be understood that the present invention
contemplates a variety of diagnostic protocols within its
10   spirit and scope.

As discussed earlier, the present invention includes
potential methods for treating lipids undergoing
glycosylation in an effort to retard if not totally
15   inhibit the progress of the Maillard and non-enzymatic
glycosylation processes. The method comprises the
development of antagonists that when administered to the
glycosylating lipid mass, serve by their structure and/or
reactivity, to inhibit rather than facilitate the
20   continued glycosylation of the lipid.

For example, the AGE-lipids of this invention can be
utilized as adjuvants due to their cross-linking
potential with antigens and also as macrophage stimulants
25   to activate the macrophage to effect removal of AGEs.
When a lipid-AGE is utilized as an adjuvant it is reacted
or cross-linked with an antigen that is "weak". The
addition of AGE-lipid to the antigen produces an antigen
which produces a strong reaction due to the presence of
30   the AGE-lipid portion, thus increasing the immunogenicity
of the original antigen. The invention is not limited to
this methodology, but rather encompasses it within its
scope.

35   As noted earlier, phagocytic cells are capable of
recognizing and removing abnormal macromolecules by means
of receptors on their surfaces which recognize specific

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chemical structures and bind them. Once the abnormal
macromolecule is recognized in this way, the phagocytic
cell may internalize the macromolecule and may then
degrade it. In some instances, the phagocytic cell may
5 in addition secrete enzymes and other factors to help
degrade the molecule or particle extracellularly if it
cannot be internalized or to produce other cells to
participate in such degradation. After the damaged
protein is removed, new growth of normal tissue can
10 ensue, and normal function of the affected area may
resume.

Phagocytic cells in the body comprise numerous types of
white blood cells. One type of white blood cell, the
15 monocyte, is produced in the bone marrow, and circulates
briefly in the blood and thereafter enters the tissues
where it becomes a macrophage.

As discussed earlier, the present invention extends to
20 the discovery that the phagocytic cells including
monocytes and macrophages can be modified by exposure to
stimulator compounds that potentiate the capability of
these cells with respect to their recognition and
affinity for, and capability to degrade advanced
25 glycosylation end products. In particular, the exposure
of these cells to certain stimulator compounds has been
found to increase the number of receptors developed on
these cells and to thereby increase the capacity and
efficiency of these cells with respect to the recognition
30 and degradation of advanced glycosylation endproducts.
The AGE-lipids of the present invention can function as
stimulator compounds.

Accordingly, the method of the present invention
35 generally comprises exposing the animal body to
stimulator AGE-lipids, which cause the body, and its
phagocytic cells in particular to become activated and to

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increase the recognition and removal of target
macromolecules that have undergone advanced
glycosylation.

5   Various methods of treatment and use are applicable
     herein. One preferred use is for the treatment or
     removal of proteinaceous or fatty deposits such as
     amyloids or lipofuscin in a mammal. The AGE-lipid, an
     antibody to AGE-lipids, or a compound which inhibits the
10   formation of AGE-lipids is administered to the mammal in
     need of such treatment in an amount effective to treat,
     remove or cause the removal of said lipofuscin.

     Another preferred use is for the treatment or prevention
15   of skin disorders, e.g., wrinkling. The AGE-lipid, an
     antibody to AGE-lipids, or a compound which inhibits the
     formation of AGE-lipids can be administered to the mammal
     in an amount effective for the treatment or prevention of
     wrinkling. All forms of administration are possible,
20   with the most preferred route of administration being
     topical application in a pharmaceutically acceptable
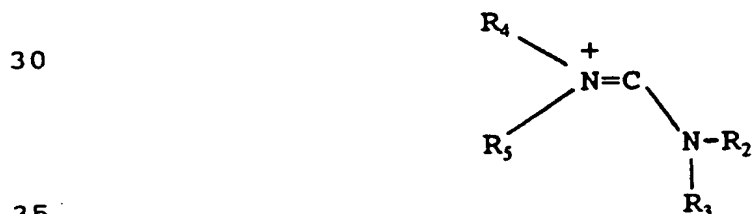   dosage form.

     Without limiting the invention to a particular mechanism
25   of action, the AGE-lipid may act directly, having a
     positive or negative metabolic effect, or indirectly,
     such as by affecting the activity of, e.g., cytokines or
     macrophage cells or immunological mediators, for
     instance, which in turn may cause the desired therapeutic
30   effect.

     Also as stated earlier, the invention extends to the
     discovery that certain compounds that have previously
     been identified as inhibitors of the advanced
35   glycosylation of proteins, also inhibit the formation of
     lipid advanced glycosylation endproducts and, further,
     react directly with malonyl-dialdehyde-like fatty acid

oxidation products. These inhibitors of advanced
glycosylation endproduct formation in proteins are
broadly set forth in U.S. Patent No. 4,758,583, the
disclosure of which is incorporated herein by reference.

5 These compounds include compounds that react with a
carbonyl moiety of an early glycosylation product.
Representative of such advanced glycosylation inhibitors
are aminoguanidine, lysine and α-hydrazinohistidine.

10 In addition to these specific compounds, other agents
capable of inhibiting the advanced glycosylation or
proteins have likewise been identified and are also
utilizable to similarly inhibit the advanced
glycosylation of lipids. These agents are set forth in

15 U.S. Patent Nos. 4,908,446; 4,983,604; 5,140,048;
5,175,192; 5,114,943; 5,137,916; 5,130,337; 5,100,919;
and 5,106,877, the disclosures of which are likewise
incorporated herein by reference.

20 Accordingly, such compounds include a variety of
hydrazine derivatives having, for example, a generic
formula as follows:

$$R - \overset{\overset{\displaystyle R_1}{|}}{N} - NH_2$$

25

wherein R is a group of the formula

30

$$\begin{array}{c} R_4 \\ \diagdown \\ \diagup \\ R_5 \end{array} \overset{+}{N} = C \begin{array}{c} \diagdown \\ N - R_2 \\ | \\ R_3 \end{array}$$

35

and R_1 is hydrogen or a lower alkyl group of 1-6 carbon
atoms, a hydroxyethyl group, or together with R_2 or R_4 may
be a lower alkylene bridge of 2-4 carbon atoms; R_2 is

40 hydrogen, amino, hydroxy, a lower alkyl group of 1-6
carbon atoms, or together with R_1 or R_3 is a lower

38

alkylene bridge of 2-4 carbon atoms; R_2 may also be an
aminoalkylene group of the formula

$$-(CH_2)_n-N-R_6$$
$$|$$
$$R_7$$
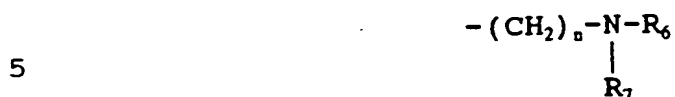
wherein n is an integer of 2-7 and R_6 and R_7 are
independently a lower alkyl group of 1-6 carbon atoms or
together form a part of a cycloalkyl or heterocyclic ring
containing from 1 to 2 heteroatoms, of which at least one
is nitrogen; and the second of said heteroatoms is
selected from the group consisting of nitrogen, oxygen,
and sulfur; with the proviso that when the second of said
heteroatoms of the heterocyclic ring is nitrogen and
forms a piperazine ring; it may be optionally substituted
by a substituent that is identical to the portion of the
compound on the first nitrogen of the piperazine ring; R_3
is hydrogen, a lower alkyl group of 1-6 carbon atoms, or
together with R_2 or R_4 is a lower alkylene bridge of 2-4
carbon atoms; R_4 is hydrogen, a lower alkyl group of 1-6
carbon atoms or together with R_1 or R_3 is a lower alkylene
bridge of 2-4 carbon atoms; or an amino group; R_5 is
hydrogen, or a lower alkyl group of 1-6 carbon atoms;
with the proviso that at least one of R_1, R_2, R_3, R_4 or R_5
is other than hydrogen; or R is an acyl or a lower
alkylsulfonyl group of up to 10 carbon atoms and R_1 is
hydrogen; and their pharmaceutically acceptable acid
addition salts.

The lower alkyl and lower alkoxy groups referred to
herein contain 1-6 carbon atoms and include methyl,
methoxy, ethyl, ethoxy, propyl, propoxy, butyl, butoxy,
pentyl, pentyloxy, hexyl, hexyloxy and the corresponding
branched chain isomers thereof.

The acyl portion referred to herein is a residue of lower
alkyl, aryl, and heteroaryl carboxylic acids containing

39

2-10 carbon atoms. They are typified by acetyl,
propionyl, butanoyl, valeryl, hexanoyl and the
corresponding higher chain and branched chain analogs
thereof. The acyl radicals may also contain one or more
5   double bonds and/or an additional acid functional group,
e.g., glutaryl or succinyl. The heteroaryl groups
referred to above encompass aromatic heterocyclic groups
containing 3-6 carbon atoms and one or more heteroatoms
such as oxygen, nitrogen or sulfur.

10

The lower alkyl sulfonyl groups of the compounds of this
invention are those containing from 1 to 7 carbon atoms
and are typified by methylsulfonyl, ethylsulfonyl, n-
propylsulfonyl, t-butylsulfonyl and the like.

15

The term "aryl" as used herein refers to phenyl and lower
alkyl substituted phenyl groups containing 6-10 carbon
atoms and substituted by one or more substituent groups
selected from among chloro, bromo, fluoro, carboxy, lower
20   alkyl, hydroxy, or lower monoalkylamino, lower
dialkylamino, and lower alkoxy.

Accordingly, where identified herein, the term
"inhibitors of advanced glycosylation" is intended to
25   encompass both the compounds such as aminoguanidine,
lysine and α-hydrazinohistidine, and other agents as
generically expressed hereinabove and as may be contained
in other related patent applications and patents issued
subsequently to U.S. Patent No. 4,983,604 and having
30   reference thereto.

In the following examples, the effect of AGE-lipids on
atherogenesis was demonstrated by the reaction of glucose
with the amine-containing lipid, phosphatidylethanolamine
35   (PE). In the presence of EDTA, a nitrogen atmosphere,
and at physiological pH and temperature, glucose (5-500
mM) reacts with PE in a time- and concentration-dependent

40

manner to form lipid-soluble products with the
spectroscopic properties of AGEs.

AGE formation induced fatty acid oxidation with reaction
5   kinetics that paralleled AGE-associated absorbance and
fluorescence. Incubation of glucose with
phosphatidylcholine (PC), in which the amine is blocked
and unable to react with glucose to initiate AGE
formation, resulted in neither spectroscopic changes nor
10 fatty acid oxidation.

Aminoguanidine prevented lipid-AGE formation and lipid
oxidation of PE. Again without limiting the invention to
a specific reaction mechanism, aminoguanidine may have
15 inhibited fatty acid oxidation by two mechanisms: first,
the formation of lipid-associated AGEs was inhibited.
Second, the direct reaction with malonyl dialdehyde-like
fatty acid oxidation products was inhibited. Lipids
containing reactive groups, e.g., primary amino groups
20 therefore react readily with reducing sugars to form AGEs
and this induces fatty acid oxidation. Aminoguanidine
inhibited the formation of lipid associated AGEs and
reacts directly with malonyl-dialdehyde like fatty acid
oxidation products.
25
Further details of the above and additional studies are
presented below.

                        EXAMPLE 1
30
To ascertain whether and to what extent lipids are
capable of reacting to form advanced glycosylation
endproducts, the lipids phosphatidylethanolamine and
phosphatidylcholine were placed into contact with a
35 sugar, preferably a reducing sugar, and allowed to
incubat together. Aliquots of the incubation mixture
were thereafter assayed as described below, for evidence

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of the presence of lipid-associated or lipid-attached
AGEs. The accumulation of AGEs indicates spontaneous
formation of AGE-lipids by non-enzymatically mediated
chemical reactions between lipid and sugar precursor
5 compounds.

Methods

Lipid in the form of phosphatidylethanolamine (PE;:L-α-
phosphatidylethanolamine, dioleoyl) (1,2-di[(cis)-9-
10 octadecenoyl]-sn-glycero-3-phosphoethanolamine) or
phosphatidylcholine (PC) (L-α-phosphatidylcholine,
dioleoyl) 1,2-di[(cis)-9-octadecenoyl]-sn-glycero-3-
phosphocholine was incubated with glucose (Glu) (L-D-
glucose) as follows: Ten mg of PE or PC in $CHCl_3$ solution
15 was evaporated to dryness. To this sample was added 1 ml
of glucose (0.5 M) in $NaPO_4$ buffer (100 mM, pH 7.4)
containing EDTA (1 mM). This solution was previously
deaerated by freeze/thawing and saturating the solution
with N_2 gas. The dried lipid was dispersed in the glucose
20 solution by immersing the sealed tube in a bath sonicator
for 30 minutes. Tubes where then placed at 37°C, in the
dark, and incubated for the indicated period of time. At
intervals, separate tubes continuing parallel incubations
(aliquots) were removed, and the lipids extracted for
25 analysis as follows.

Aliquots were shaken with 1 ml of chloroform/methanol
(2:1) for ten minutes. After removal of the organic
layer, this extraction was repeated twice. The organic
30 layer was then back extracted twice with ice cold,
deaerated H_2O. After evaporation, lipids were redissolved
in chloroform/methanol (1:1) and analyzed by absorption
spectroscopy ($OD_{200-800}$ or OD_{360}); or fluorescence
spectroscopy (excitation wavelength (Ex) 360 nm, emission
35 wavelength (Em) 440 nm).

42

The results are shown in Figures 1 and 2. Figure 1 shows
the time dependent increase in absorbency at 360 nm,
which absorbency is characteristic of AGEs and AGE-
containing compounds. As shown Figure 1, incubation of
5   PE with glucose leads to a significant accumulation of
material with AGE-typical absorbance, but incubation of
the PC with glucose produces almost none. This result
suggests that the free amino group represented in PE may
be required for AGE formation while the blocked amino
10   group of PC lacks the reactivity to make AGE formation
possible.

Figure 1 indicates that formation of OD_{360} material depends
on glucose concentration; less AGE-specific absorbance is
15   found after incubation of PE with 50 mM glucose than
after parallel incubations with 500 mM glucose.

Figure 1 also indicates that aminoguanidine, an inhibitor
of AGE formation on proteins, inhibits AGE-lipid
20   formation as indicated by lower AGE-specific OD_{360} values
in incubations for PE with 0.5 M glucose and 0.1 M
aminoguanidine than in parallel incubations of PE and
glucose without aminoguanidine.

25   Figure 2 shows parallel measurements of fluorescence
intensity on the same sets of samples. The results and
conclusions are as above. AGE-lipids form spontaneously
in incubations of PE and glucose, but not in incubations
of PC and glucose. AGE-lipid formation is glucose
30   concentration-dependent, and aminoguanidine inhibits AGE-
lipid formation as monitored by the accumulation of AGE-
typical fluorescence material.

Figure 4 shows the changes in the ultraviolet and visible
35   absorbance spectrum (200-500 nm) that occur over time
when PE is incubated with 0.5M glucose as described
above. These changes are characteristic of the formation

43

of AGEs and AGE-like chromophores. Figure 4 shows that
more AGE-typical UV absorbance occurs with progressively
longer incubations.

5   Figures 5 and 6 show the dependence of AGE-lipid
formation on glucose concentration in the incubation
mixture. Incubation of PE with glucose for 50 days leads
to AGE-specific absorbance (Figure 5); and fluorescence
(Figure 6). The glucose concentration dependence of
10 lipid oxidation is shown in Figure 7.

EXAMPLE 2

To demonstrate that AGE-lipid formation is associated
15 with changes in lipid oxidation, the accumulation of
malonyldialdehyde (MDA)-like oxidation products was
measured in samples from the incubations described above.

Methods
20 Lipid peroxidation products were quantified by the
thiobarbituric acid reactive substances methods (TBARS;
PROC. NATL. ACAD. SCI. USA **81**:3883-3887). Briefly, 0.1
ml of lipid extract was added to 0.2 ml of TBA reagent
(0.37% thiobarbituric acid, 10% trichloroacetic acid) and
25 heated to 100°C for 30 minutes. n-Butanol extractable
material (1 ml) was then analyzed by fluorescence
spectroscopy (emission at 553 nm upon excitation at 515
nm). Thiobarbituric acid-reactive substances were
quantitated by comparison of duplicate experimental
30 samples to an MDA standard curve that was obtained by
assaying in duplicate, 0.1-20 nmole of MDA. Oxidative
modification values are expressed as MDA equivalents
(pmMDA(eq)/μg lipid).

35 ## Results
Figures 3 and 7 show that lipid oxidation increased in
conjunction with AGE-lipid formation. Specifically,

44

incubation of PE with glucose led to lipid oxidation in a time- (Figure 3) and glucose concentration- (Figure 7) dependent fashion. Incubations of PC with glucose led to little or no lipid oxidation (Figure 3), and

5   aminoguanidine inhibited oxidation of lipid in PE/glucose incubations (Figure 3).

EXAMPLE 3

10   The formation of AGEs on lipids was additionally assessed by AGE-specific ELISA.

Methods

Lipids (PE or PC) were incubated with glucose as

15   described above. AGE content was quantitated by ELISA using a specific anti-AGE antibody (see Makita et al., *J. BIOL. CHEM.* (1992), **267**:5133-5138). Protein-linked AGEs were measured by competitive ELISA utilizing an AGE standard synthesized by incubation of glucose with BSA.

20   In this ELISA, 1.0 U of AGE activity is defined as the amount of antibody-reactive material that is equivalent to 1.0 μg of AGE-BSA standard. Lipid-derived AGEs were measured in a direct, non-competitive ELISA as follows. For each sample, triplicate 100 μl aliquots of lipid-

25   soluble material (dissolved in methanol) were added to round-bottom, 96 well plates and the solvent evaporated. The wells then were washed three times with PBS/0.05% Tween-20. Antiserum (final dilution 1/1000) was added, the plates were incubated for 1 hour at room temperature,

30   and the wells washed and processed as described for the competitive ELISA. Control samples were developed with pre-immune serum in place of anti-AGE antiserum. Results were quantitated with reference to a standard curve that was obtained by assaying dilutions of AGE-BSA standard

35   that were absorbed to plates in a concentration range from 0.3 ng/ml to 3 μg/ml.

45

<u>Results</u>

Table 1 shows the formation of AGE-lipids from
phosphatidylethanolamine versus the control lipid
phosphatidylcholine (in which the amino group is blocked
5 and thus thought to be prevented from reacting with the
reducing sugar glucose).

<u>TABLE 1</u>

10 Incubation Time	AGE, Units/mg lipid	
Days	PE + glucose	PC + glucose
0	<0.005	<0.005
10	0.024±0.003	<0.005
20	0.20± 0.03	<0.005
30	0.13± 0.02	<0.005
40	0.15± 0.02	<0.005
50	0.13± 0.02	<0.005

20

<u>EXAMPLE 4</u>

25 <u>Reaction between aminoguanidine and malonyldialdehyde</u>

Example 2 showed that AGE-lipid formation is accompanied
by a parallel increase in lipid oxidation as measured by
an increase in the concentration of MDA-like substances.
30 It is notable that this oxidation of lipids occurred
without added metals, such as copper, which are commonly
employed to initiate lipid oxidation. Additionally, the
above examples indicate that aminoguanidine prevents both
AGE-lipid formation and lipid oxidation.

35

To demonstrate that aminoguanidine can react directly
with MDA-like aldehydes to prevent their reaction with
proteins, aminoguanidine was incubated with MDA.

40 <u>Methods</u>

Malonyldialdehyde (MDA) standard solutions (0.5 ml) were
prepared by dilution of malonaldehyde bis(diethyl acetal)

46

into H_2O. Aminoguanidine HCl (0.5 ml) was added, followed by 0.2 ml of the TBA reagent described above. The solution was incubated at room temperature for 10 minutes and TBARS measured by specific fluorescence as described

5   hereinabove.

Results

The results are shown in Figures 8A and B. Figure 8A shows the progressive inhibition of thiobarbiturate

10 reactivity of MDA (indicated by relative fluorescence) by increasing amounts of aminoguanidine. Thus, aminoguanidine may inhibit protein modification by lipid oxidation products by quenching reactive aldehydes before the latter can participate extensively in subsequent

15 modification of protein amino groups.

Figure 8B plots one of the latter data sets from Figure 8A, illustrating the increase in inhibition (A) of the fixed initial amount (10 mM MDA) of thiobarbiturate

20 activity by increasing concentrations of added aminoguanidine.

EXAMPLE 5

25 To define further the relationship between advanced glycosylation and LDL oxidation *in vivo*, LDL was isolated from plasma obtained from either non-diabetic or diabetic individuals and analyzed for the presence of lipid-AGEs, apoprotein-AGEs, and oxidative modification. These

30 specimens were obtained from 8 normoglycemic, non-diabetic controls and 16 patients with Type I or Type II diabetes mellitus.

Methods

35 Plasma LDL (d = 1.025-1.063 g/ml) was isolated from healthy, non-hyperglycemic individuals and patients with diabetes mellitus by sequential ultracentrifugation,

using 2.7 mM EDTA. The isolated and re-centrifuged LDL
was dialyzed extensively against PBS containing 2.7 mM
EDTA and 0.2 mM BHT. LDL was sterile filtered before
further use and the protein content determined by the

5   Lowry method. The non-diabetic patient group (n = 8) had
a mean age of 34.6 ± 9.6 years. The diabetic group (n =
16) consisted of 5 patients with Type I diabetes and 11
patients with Type II diabetes. The mean age was 55.5 ±
16.3 years and the mean duration of diabetes was 11.9 ±

10 5.6 years. The mean hemoglobin A_{1c} level was 10.0% ±
1.7%. The P values were calculated by the unpaired
Student's *t*-test statistic for comparison between groups.

Results

15 The LDL was analyzed for oxidative modification and then
fractionated into lipid and apoprotein components for
AGE-ELISA measurements (Figure 9). In agreement with
prior studies, LDL from diabetic individuals was observed
to have undergone significantly greater oxidative

20 modification than the LDL from non-diabetic individuals
[Normal, Non-diabetics (NL): (n=8) 3.7 ± 1.25 pm MDA
equivalents/μg LDL; Diabetics (DM): (n=16) 6.8 ± 1.2 pm
MDA equivalents/μg LDL (Mean ± SD), P< 0.0001)]. Of
significance, both the lipid- and the apoprotein-linked

25 AGEs in the diabetic LDL specimens were found to be
markedly elevated when compared to the LDL specimens
obtained from non-diabetic individuals. Lipid-AGE levels
were elevated almost 4-fold in diabetic patients [Normal,
non-diabetic (NL): (n=8) 0.11 ± 0.03 Units of AGE/μg

30 lipid; Diabetics (DM): (n=16) 0.41 ± 0.25 Units of AGE/μg
lipid, P<0.005)]. Apoprotein-AGE levels were increased
approximately 2-fold in the diabetic samples [NL: (n=8)
0.0028 ± 0.0006 Units of AGE/μg apoprotein; DM: (n=16)
0.0068 ± 0.004 Units of AGE/μg apoprotein, P<0.0001)].

35

These measurements revealed a similar quantitative ratio
between LDL oxidation and the level of AGE-lipid and AGE-

48

apoprotein that was similar to that observed during LDL
incubation *in vitro* (Figure 10). There also appeared to
be a marked increase in the level of lipid-AGEs relative
to the level of apoprotein-associated AGEs. Linear
5   regression analysis of these data revealed significant
correlation between the level AGE modification and LDL
oxidation. For the measurement of AGE-apoprotein versus
LDL oxidation, this analysis showed a correlation
coefficient of r=0.52 and P<0.01. For AGE-lipid versus
10 LDL oxidation, the corresponding values were r=0.63 and
P<0.005.

EXAMPLE 6

15 It was also postulated that contiguous basic residues
(Arg-Lys-Arg) within the receptor-binding domain of apo B
might serve as a reactive site for AGE formation, thus
preventing normal pathways of LDL clearance. To begin to
address this hypothesis, LDL levels were examined in 10
20 diabetic patients enrolled in a 28-day trial of
aminoguanidine (AG), a pharmacological inhibitor of
advanced glycosylation.   •

LDL levels in 10 diabetic patients enrolled in a 28-day
25 trial of aminoguanidine (AG) were measured by the AGE-
specific ELISA of Example 3. The efficacy of AG therapy
was assessed by reduction in the level of hemoglobin-AGE
(Hb-AGE), a circulating marker of advanced glycosylation.
The results are given in Figure 14 and Table 2, below.
30 Table 2 also includes additional data relating to Hb-AGE.

TABLE 2

Patient Group	Hb-AGE	LDL
Placebo	8.9 ± 0.6% reduction	2.0 ± 0.2% reduction

49

AG
treatment   27.5 ± 1.6% reduction   29.8 ± 3.1% reduction
              Mean ± SD, p <0.0025   Mean ± SD, p <0.002

5   Inhibition of AGE formation was associated with a 30%
    decrease in LDL levels, as illustrated in Figure 14.

    This study suggests that the advanced glycosylation may
    account for elevated LDL levels, and that aminoguanidine
10 therapy may serve to improve LDL clearance and diminish
    the risk of atherogenesis in diabetic patients.

EXAMPLE 7

15 To determine whether human AGE-peptides can react with
    plasma lipoproteins, AGE-peptides isolated from diabetic
    sera were incubated with human LDL, in the presence or
    absence of the AGE-inhibitor aminoguanidine (300 mM) for
    14 days and the results compared to controls (LDL only)
20 and a parallel incubation of LDL with glucose. AGE
    levels in apo B and in lipid fractions of LDL were
    measured by the AGE-specific ELISA procedure of Example
    3, and the results given below in Table 3, and in Figures
    11A and 11B. In addition, LDL oxidation was measured as
25 in Example 4, and the AGE-formation was found to parallel
    LDL-oxidation.

    As shown in Table 3 below, marked increases in AGE
    content of apo B, and of lipid fraction of LDL were noted
30 as a function of time compared to control samples. These
    values far exceeded those obtained using glucose. AGE-
    formation paralleled LDL oxidation. In the presence of
    the AGE-inhibitor aminoguanidine, AGE-formation, as well
    as lipid oxidation, was markedly inhibited.

35
    In conclusion, circulating AGE-peptides are an important
    in vivo source of AGE-lipids, and oxidative modification

of plasma LDL, in excess of and independent of glucose. Aminoguanidine may be of therapeutic benefit in diabetics, where elevated AGE-peptide levels and hyperlipidemia may be causally linked to accelerated

5 atherosclerosis.

TABLE 3

Sample	AGE Level		Oxidized LDL Level
Control (LDL only)	Apo B	2.0 AGE U/mg	3 nmoles MDA/mg
	lipid	130 AGE U/mg	
Glucose (100 mM) + LDL	Apo B	7 AGE U/mg	
	lipid	630 AGE U/mg	
10 LDL + AGE-peptides	Apo B	47 AGE U/mg	56 nmoles MDA/mg
	lipid	2200 AGE U/mg	
LDL + AGE-peptides	Apo B	7 AGE U/mg	12 nmoles MDA/mg
+ AG	lipid	290 AGE U/mg	

52

EXAMPLE 8

The AGE-specific ELISA of Example 3 was used to measure
AGE moieties attached to the apo B and lipid components
5   of LDL isolated from normal controls (n=17) and diabetic
patients (n=43). The results are shown below in Table 4.
Additional data reflecting the study of lipid oxidation
in these patient samples are presented in Figures 12A-
12C.

10

TABLE 4

Patient Type	AGE level (lipid)	AGE level ApoB
5 Control	109 AGE U/mg	2.9 AGE U/mg
Diabetic patients with none or single complication	317 AGE U/mg $p < 0.005$	4.6 AGE U/mg $p < 0.001$
10		
Diabetic patients with multiple complications	552 AGE U/mg $p < 0.001$	8.4 AGE U/mg $p < 0.001$
15 Diabetic patients with severe diabetic nephropathy (ESRD)	3270 AGE U/mg $p < 0.001$	71 AGE U/mg $p < 0.001$

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These data indicate that circulating AGE-LDL levels
correlate closely with the number and severity of
diabetic complications and support an etiopathological
relationship between AGE-modification and both the micro-
5 and macro-vascular complications of diabetes since LDL
from diabetic patients with multiple complications
contained five-fold higher levels of AGE-lipid and almost
three-fold higher levels of AGE-apo B when compared to
normal controls. Diabetics with severe diabetic
10 nephropathy (ESRD) showed marked elevation of AGE-
modified LDL when compared to diabetic patients without
renal involvement.

The results also indicate the AGE-lipid modification was
15 associated with proportionally increased lipid oxidation
within each group of patients (non-diabetic: 3.7
nmoles/mg; (0-1 complications): 5.2   nmoles/mg (>4
complications): 8.4 nmoles/mg [measured as nmole MDA
equiv/mg LDL].
20
                          EXAMPLE 9

An additional patient population was examined to provide
data cumulative with the data collected and evaluated in
25 Example 8, above. Accordingly, to determine levels of
circulating AGE-low density lipoprotein, apoprotein B,
plasma LDL was collected from the selected patients as
described in Example 8. Briefly, LDL was delipidated
with methanol-ether 1:3 (V/V). Apo B was digested by
30 incubating at 37°C for 24 hours by using Proteinase K,
(1 mg/ml). These samples were inactivated by heating at
70°C for 1 hour. AGE levels were determined by ELISA as
described in Example 3. The results are presented in
Figure 13.
35
To obtain a measure of the *in vivo* "reactivity" of AGE-
peptides, AGE levels were determined on a short-lived

55

plasma protein, apoprotein B, in patients with and
without diabetes. As shown in Figure 13, marked
elevations of AGE-Apo B were found in LDL from diabetic
(mean 69.4 ± 21.7 AGE U/mg, p<0.0001), as well as non-
5   diabetic patients with ESRD (mean 27.6 ± 16.5 AGE U/mg,
p<0.0001) compared to normals (2.8 ± 0.5 AGE U/mg), and
diabetics with normal renal function (mean 3.9 ± 1.0 AGE
U/mg). Since non-diabetics with ESRD are normoglycemic,
with normal levels of HbA_{1c}, the data clearly suggest that
10 glucose is not the only source of AGE modification. This
is confirmatory of the observation noted in Example 8,
above.

DISCUSSION

15 The data presented here provide an important link between
elevated levels of plasma AGEs and accelerated
vasculopathy associated with ESRD, consisting of the
striking ability of endogenous AGE-peptides to "react"
with key proteins, such as collagen and lipoproteins.
20 LDL apoprotein B is a short-lived plasma protein, long
implicated in atherogenesis. The efficient generation of
AGE-apo B by AGE-peptides *in vivo* suggested a possible,
in vivo marker for the "toxicity" of AGE-peptides. The
patient data on circulating AGE-apo B supported this
25 notion.

The level of AGE modification of plasma LDL/Apo B in
patients with ESRD was significantly elevated compared to
the level of the normal subjects (ten-fold for non-
30 diabetic (ESRD) and twenty-five fold for diabetic (ESRD)
patients). Given the relatively short half-life of the
LDL particle in blood, the degree of AGE modification in
ESRD patients cannot be attributed solely to ambient
glucose or other "intermediate" glycosylation products
35 found in plasma. The contribution of these agents in the
non-diabetic ESRD patients is also improbable given the
normal glucose and HbA1c levels in these non-diabetic

56

patients.   Instead, a principal role can be attributed to
the so-called "AGE-peptides", or low molecular weight
blood-borne AGE-modified species. This is supported by
the relatively elevated levels of AGE-peptide in ESRD
5   patients, whether diabetic or not. Further support is
for this notion is provided by the relatively modest
elevations of AGE-Apo B in diabetic patients with normal
renal function, despite their hyperglycemia as reflected
in elevated HbA_{1c}.

10

This interpretation is consistent with the argument that
the absence of renal function plays a more important role
in total serum AGE accumulation than an increased rate of
AGE formation due to hyperglycemia. It is further
15   supported by the findings indicating the clearance of
serum AGEs by the kidneys, as measured by urinary
excretion, does not differ significantly between normal
and diabetics, as long as renal function is preserved.

20   The following publications relate generally to advanced
glycosylation endproducts and the reactions in which such
products are involved.

"Function of Macrophage Receptor for Nonenzymatically
25   Glycosylated Proteins is Modulated by Insulin Levels,"
Vlassara, Brownlee and Cerami, *DIABETES* (1986), Vol. 35
Supp. 1, Page 13a;

"Accumulation of Diabetic Rat Peripheral Nerve Myelin by
30   Macrophages Increases with the Presence of Advanced
Glycosylation Endproducts," Vlassara, H., Brownlee, M.,
and Cerami, A. *J. EXP. MED.* (1984), 160:197-207;

"Recognition and Uptake of Human Diabetic Peripheral
35   Nerve Myelin by Macrophages," Vlassara, H., Brownlee, M.,
and Cerami, A. *DIABETES* (1985), 34(6):553-557;

57

"High-Affinity-Receptor-Mediated Uptake and Degradation
of Glucose-Modified Proteins: A Potential Mechanism for
the Removal of Senescent Macromolecules," Vlassara H.,
Brownlee, M., and Cerami, A., *PROC. NATL. ACAD. SCI. USA*
5   (Sept. 1985), **82**:5588-5592;

"Novel Macrophage Receptor for Glucose-Modified Proteins
is Distinct from Previously Described Scavenger
Receptors," Vlassara, H., Brownlee, M., and Cerami, A.
10 *J. EXP. MED.* (1986), **164**:1301-1309;

"Role of Nonenzymatic Glycosylation in Atherogenesis,"
Cerami, A., Vlassara, H., and Brownlee, M., *J. CELL.*
BIOCHEMISTRY (1986), **30**:111-120;

15
"Characterization of a Solubilized Cell Surface Binding
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Nonenzymatically by Advanced Glycosylation Endproducts,"
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5　This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended Claims, and 10　all changes which come within the meaning and range of equivalency are intended to be embraced therein.

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WHAT IS CLAIMED IS:

1   1.   A method of modulating lipid oxidation in a mammal
2   comprising administering to said mammal a lipid
3   oxidation-modulating effective amount of an agent capable
4   of controlling the formation of AGEs in said mammal.

1   2.   The method of Claim 1 comprising a method for
2   inhibiting said lipid oxidation, wherein said agent is
3   capable of inhibiting the formation of said AGEs.

1   3.   The method of either of Claims 1 or 2, wherein said
2   AGEs comprise AGE-lipids.

1   4.   The method of Claim 3 wherein said AGE-lipids are
2   formed from lipid-related materials, and said lipid-
3   related materials are selected from amine-containing
4   lipids, low-density lipoproteins, and apolipoproteins.

1   5.   The method of Claim 4, wherein said apolipoproteins
2   comprise apolipoprotein B (apo B).

1   6.   The method of Claim 2, comprising a method for
2   lowering low-density lipoprotein levels in said mammal.

1   7.   The method of Claim 2, comprising a method for the
2   prevention and/or treatment of end stage renal disease.

1   8.   The method of Claim 2, comprising a method for the
2   prevention and/or treatment of atherosclerosis.

1   9.   The method of Claim 2, comprising a method for the
2   prevention and/or treatment of hypercholesterolemia.

1   10.   A method of modulating lipid metabolism in a mammal
2   in need of such treatment comprising administering to
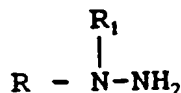3   said mammal a lipid metabolism-modulating effective

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4 amount of an agent capable of modifying the recognition
5 and removal of lipids and lipid-related materials from
6 serum, wherein said agent is capable of controlling the
7 formation, accumulation and/or activity of AGEs.

1 11.   The method of Claim 10, wherein said agent is
2 selected from the group consisting of antibodies against
3 advanced glycosylation endproducts, ligands capable of
4 binding and/or neutralizing advanced glycosylation
5 endproducts, receptors for advanced glycosylation
6 endproducts, and compounds capable of inhibiting the
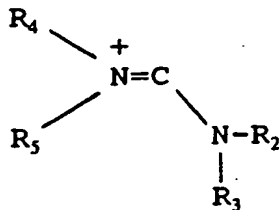7 formation of advanced glycosylation endproducts.

1 12.   The method of Claim 11, wherein said compounds
2 capable of inhibiting the formation of advanced
3 glycosylation endproducts are capable of reacting with an
4 active carbonyl moiety on an early glycosylation product.

1 13.   The method of Claim 12, wherein said compound is
2 selected from the group consisting of aminoguanidine, α-
3 hydrazinohistidine, lysine, an analog of aminoguanidine,
4 and mixtures thereof.

1 14.   The method of Claim 13, wherein said analog is
2 selected from the group consisting of hydrazine
3 derivatives of the formula:

$$\begin{array}{c} R_1 \\ | \\ R - N - NH_2 \end{array}$$

7 wherein R is a group of the formula

$$\begin{array}{c} R_4 \\ \diagdown \\ \quad\;\; N=C \overset{+}{\diagup} \diagdown \\ R_5 \diagup \qquad\quad N - R_2 \\ \qquad\qquad\quad | \\ \qquad\qquad\quad R_3 \end{array}$$

15 and

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16   R_1 is hydrogen or a lower alkyl group of 1-6 carbon

17   atoms, a hydroxyethyl group, or together with R_2 or R_4 may

18   be a lower alkylene bridge of 2-4 carbon atoms;

19   R_2 is hydrogen, amino, hydroxy, a lower alkyl group

20   of 1-6 carbon atoms, or together with R_1 or R_3 is a lower

21   alkylene bridge of 2-4 carbon atoms; R_2 may also be an

22   aminoalkylene group of the formula

23                  $-(CH_2)_n-N-R_6$

24                            $|$

25   R_7

26   wherein n is an integer of 2-7 and R_6 and R_7 are

27   independently a lower alkyl group of 1-6 carbon atoms or

28   together form a part of a cycloalkyl or heterocyclic ring

29   containing from 1 to 2 heteroatoms, of which at least one

30   is nitrogen; and the second of said heteroatoms is

31   selected from the group consisting of nitrogen, oxygen,

32   and sulfur; with the proviso that when the second of said

33   heteroatoms of the heterocyclic ring is nitrogen and

34   forms a piperazine ring; it may be optionally substituted

35   by a substituent that is identical to the portion of the

36   compound on the first nitrogen of the piperazine ring;

37   R_3 is hydrogen, a lower alkyl group of 1-6 carbon

38   atoms, or together with R_2 or R_4 is a lower alkylene

39   bridge of 2-4 carbon atoms;

40   R_4 is hydrogen, a lower alkyl group of 1-6 carbon

41   atoms or together with R_1 or R_3 is a lower alkylene bridge

42   of 2-4 carbon atoms; or an amino group;

43   R_5 is hydrogen, or a lower alkyl group of 1-6 carbon

44   atoms; with the proviso that at least one of R_1, R_2, R_3, R_4

45   or R_5 is other than hydrogen; or R is an acyl or a lower

46   alkylsulfonyl group of up to 10 carbon atoms and R_1 is

47   hydrogen; and their pharmaceutically acceptable acid

48   addition salts.

1   15.   A method of modulating lipid metabolism in a mammal

2   in need of such treatment comprising administering to

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3 said mammal a lipid metabolism-modulating effective
4 amount of an agent capable of modifying the recognition
5 and removal of lipids and lipid-related materials from
6 serum, wherein said agent is capable of inhibiting the
7 blockade of the LDL receptor binding domain of apo B by
8 an advanced glycosylation endproduct.

1 16.   The method of Claim 15, wherein said agent is
2 capable of preferentially binding to advanced
3 glycosylation endproducts.

1 17.   The method of Claim 16, wherein said agent is
2 selected from the group consisting of an antibody to
3 advanced glycosylation endproducts, and a receptor for
4 advanced glycosylation endproducts.

1 18.   The method of Claim 15, wherein said LDL receptor
2 binding domain of apo B contains at least one active site
3 amenable to the formation of an advanced glycosylation
4 endproduct, and said agent is capable of inhibiting said
5 advanced glycosylation endproduct from forming at said
6 site.

1 19.   The method of Claim 18, wherein said agent is
2 capable of inhibiting the formation of said advanced
3 glycosylation endproduct at said site by preferentially
4 binding to said site.

1 20.   A method for estimating the course and extent of *in*
2 *vivo* lipid oxidation in a mammal comprising measuring the
3 presence and amount of AGE-lipids in said mammal.

1 21.   The method of Claim 20, wherein said AGE-lipids are
2 measured by an *in vitro* procedure.

1 22.   The method of Claim 20, comprising the steps of:

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2   A.   preparing at least one biological sample
3   taken from said mammal in which said AGE-lipids are
4   suspected to be present;
5   B.   immobilizing a material selected from the
6   group consisting of the biological sample of Step A. and
7   a binding partner to AGE-lipid or AGE-bearing lipid-
8   related material, on a suitable substrate, and labeling
9   one of the materials not immobilized;
10   C.   incubating said sample with said binding
11   partner for a period of time sufficient for said binding
12   partner to become bound to any AGE-lipid or AGE-bearing
13   lipid-related material present in said sample;
14   D.   removing any unbound material from Step
15   C.; and
16   E.   comparing the amount of label bound to
17   said sample to a standard.

1   23.   The method of Claim 22, wherein said binding partner
2   is selected from the group consisting of a receptor for
3   an AGE, and an antibody reactive with or capable of
4   binding to said AGE-lipid.

1   24.   The method of Claim 23, wherein said antibody to
2   said AGE-lipid is selected from the group consisting of
3   polyclonal antibodies, monoclonal antibodies, and
4   chimeric antibodies.

1   25.   The method of Claim 20 comprising a method for
2   monitoring the level of low density lipoprotein in said
3   mammal.

1   26.   The method of Claim 20, comprising a method for
2   diagnosing the likelihood or onset, and/or for monitoring
3   the course and severity of a pathology selected from
4   atherosclerosis, vascular disease, diabetes, diabetic
5   nephropathy, and hypercholesterolemia.

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1 27. A method of testing the ability of a drug or other
2 entity to prevent blockade of the LDL receptor binding
3 domain on apo B, which comprises disposing a quantity of
4 apo B with unbound LDL receptor binding domain in a
5 suitable medium, simultaneously adding a quantity of an
6 material capable of forming an AGE on said binding
7 domain, and the drug under test, and thereafter measuring
8 the quantity of AGE-apo B, if any, that is formed.

1 28. An AGE-lipid comprised of the reaction product of a
2 lipid containing an AGE-reactive group reacted with an
3 advanced glycosylation endproduct or a compound which
4 forms advanced glycosylation endproducts.

1 29. An AGE-lipid according to Claim 28 wherein the AGE-
2 reactive group of the lipid is a primary amino group.

1 30. An AGE-lipid according to Claim 29 wherein the lipid
2 portion is selected from the group consisting of amine-
3 containing lipids, lipoproteins, and apolipoproteins.

1 31. An AGE-lipid according to Claim .28 wherein the
2 advanced glycosylation endproduct or a compound which
3 forms advanced glycosylation endproducts is or derives
4 from a reducing sugar.

1 32. An AGE-lipid according to Claim 31 wherein the
2 compound which forms advanced glycosylation endproducts
3 is selected from the group consisting of glucose,
4 fructose, ribose and glucose-6-phosphate.

1 33. A method of preparing an AGE-lipid comprising
2 incubating a lipid or lipid-related material containing
3 an AGE-reactive group with an advanced glycosylation
4 endproduct or a compound which forms advanced
5 glycosylation endproducts.

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1   34.   The method of Claim 33 wherein the lipid or lipid-
2   related material is selected from the group consisting of
3   amine-containing lipids, lipoproteins., and
4   apolipoproteins.

1   35.   A pharmaceutical composition comprised of an AGE-
2   lipid according to Claim 28 in combination with a
3   pharmaceutically acceptable carrier.

1   36.   A pharmaceutical composition according to Claim 35
2   wherein the AGE-lipid is selected from the group
3   consisting of AGE-phospholipids, AGE-low density
4   lipoproteins, and AGE-apolipoproteins.

1   37.   A pharmaceutical composition according to Claim 35
2   wherein the AGE-lipid or pharmaceutically acceptable
3   carrier is effective for transdermally delivering a
4   therapeutically active compound.

1   38.   A pharmaceutical composition according to Claim 35
2   suitable for topical, oral or injectable administration.

1   39.   A pharmaceutical composition according to Claim 37
2   in the form of a liposome.

1   40.   A method of rejuvenating the skin of a mammal
2   comprising administering to said mammal an effective
3   amount of the AGE-lipid according to Claim 28.

1   41.   A method according to Claim 40 wherein the AGE-lipid
2   is selected from the group consisting of AGE-
3   phospholipids, AGE-low density lipoproteins, and AGE-
4   apolipoproteins.

1   42.   A method according to Claim 40 wherein the AGE-lipid
2   is effective for rejuvenating the skin upon topical
3   administration.

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1 43.   A method of delivering an advanced glycosylation
2 endproduct to a biologically active site in a mammal,
3 comprising administering to said mammal the AGE-lipid of
4 Claim 28.

1 44. A method of delivering a drug to a biologically
2 active site having at least one AGE receptor comprising
3 combining said drug with the AGE-lipid of Claim 28 and
4 administering the drug and AGE-lipid to a mammal in an
5 amount effective for delivering said drug to said
6 biologically active site.

1 45.   A method of detecting spoilage in a lipid-containing
2 food, comprising
3   combining a sample of said food with an
4 antibody which recognizes an AGE-lipid to detect the
5 presence or amount of AGE-lipids in said sample, and
6   comparing the amount of AGE-lipids detected to
7 a standard.

1 46.   A method for removing AGE-lipids from the body of a
2 mammal comprising administering to said mammal the anti-
3 antibody or second binding partner to an AGE-lipid to
4 form an immune complex activating the animal's cellular
5 clearance system (macrophages) to remove said immune
6 complex and associated AGEs (advanced glycosylation
7 endproducts).

1 47.   A method according to Claim 46 wherein the AGE-lipid
2 is selected from the group consisting of AGE-
3 phospholipids, AGE-low density lipoproteins, and AGE-
4 apolipoproteins.

1 48.   A composition for promoting the sequestration and
2 removal from the body of an animal of target
3 macromolecules that have undergone advanced glycosylation
4 comprising an AGE-lipid capable of causing the body to

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5 increase its activity of recognizing and removing said
6 macromolecules.

1 49. A test kit to be used for the detection and/or
2 determination of one of the components selected from the
3 group consisting of AGE-lipids, and the specific binding
4 partners thereto, according to a predetermined protocol.

1 50. A test kit according to Claim 49, comprising:
2   a.   a labeled component which has been obtained by
3 coupling an AGE-lipid to a detectable label;
4   b.   one or more additional immunochemical reagents
5 of which at least one reagent is a binding partner or an
6 immobilized binding partner, which binding partner is
7 selected from the group consisting of:
8   i.   a binding partner capable of binding with
9 the labeled component (a);
10   (ii) a binding partner capable of binding with
11 a binding partner of the labeled component (a);
12   (iii) a binding partner capable of binding with
13 at least one of the component(s) to be determined; and
14   (iv) a binding partner capable of binding with
15 at least one of the binding partners of at least one of
16 the component(s) to be determined; and
17   c.   directions for the performance of a protocol
18 for the detection and/or determination of one or more
19 components of an immunochemical reaction between AGE-
20 lipid and a specific binding partner thereto.

1 51. A test kit according to Claim 49 wherein the AGE-
2 lipid is selected from the group consisting of AGE-
3 phospholipids, AGE-low density lipoproteins, and AGE-
4 apolipoproteins.

1 52. A method of detecting the presence or onset of
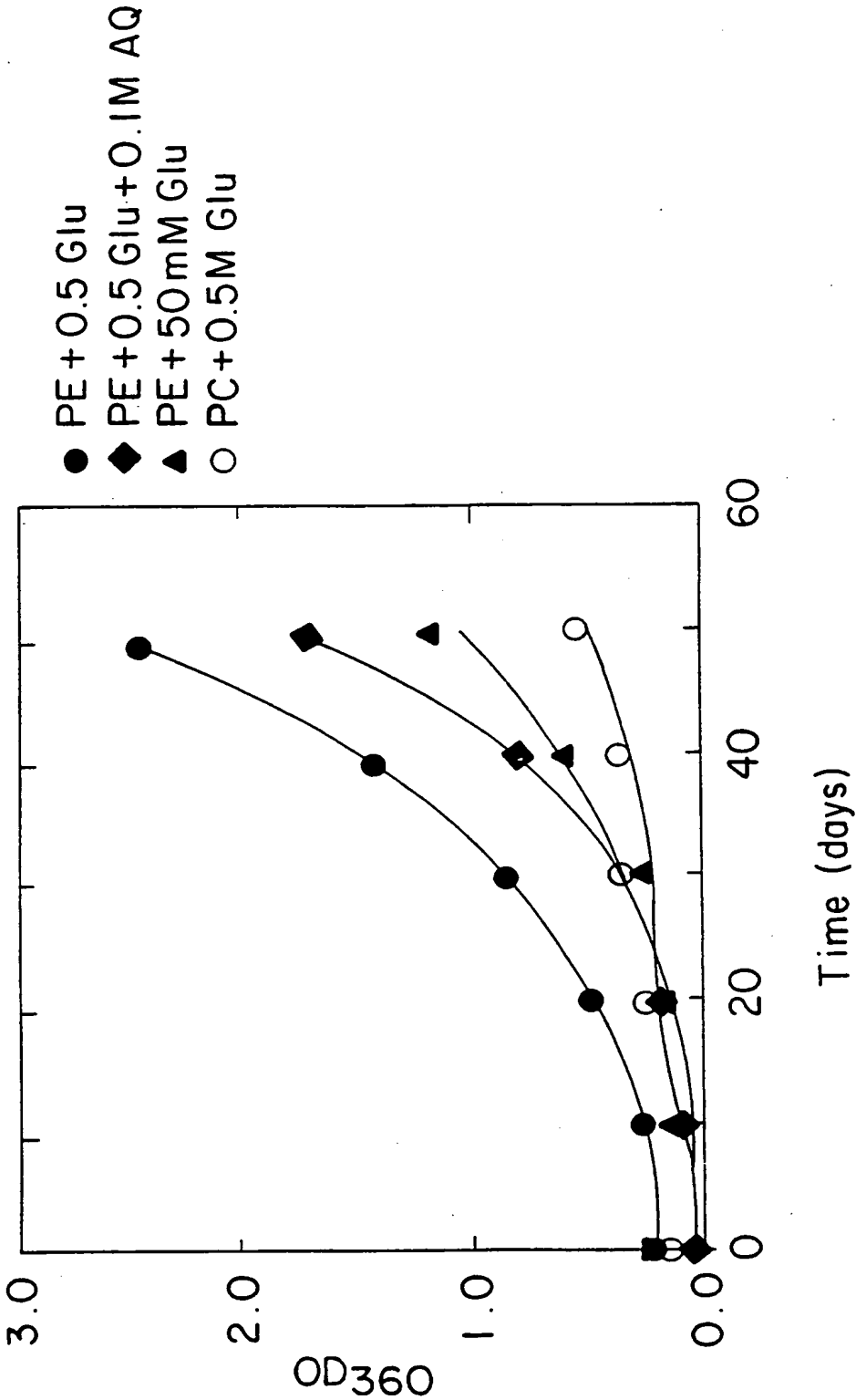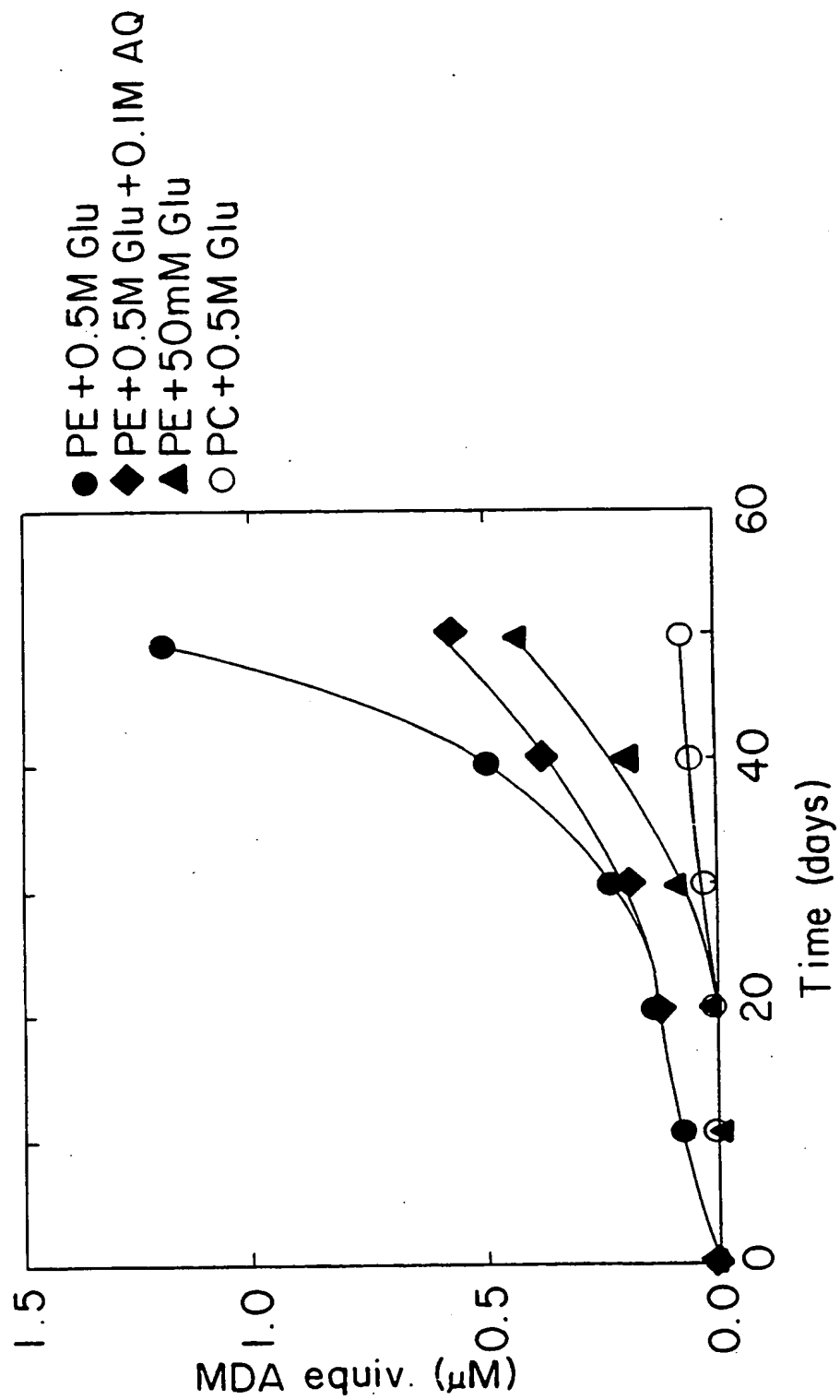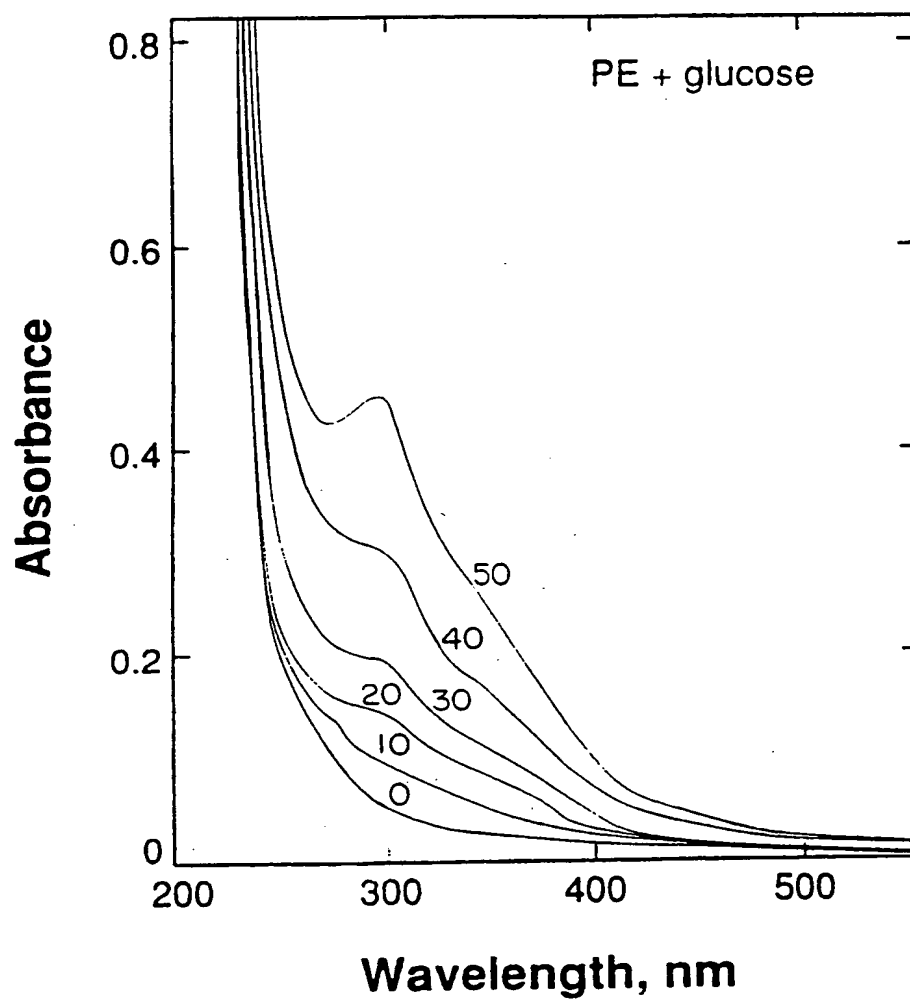2 diabetic complications which comprises measurement in a

3   patient in which such a condition is suspected of the

4   AGE-low-density lipoprotein levels.

1   53.   A method of detecting the presence or onset of

2   atherosclerosis or other related condition which

3   comprises measurement in a patient in which such a

4   condition is suspected of the AGE-low-density lipoprotein

5   levels.

1   54.   A method of inhibiting the formation of oxidized

2   lipids in a patient in need of such therapy comprising

3   administering to said patient an effective amount of

4   aminoguanidine or an analog thereof.

1   55.   The method of Claim 54 wherein said analog is

2   selected from the group consisting of lysine, α-

3   hydrazinohistidine and hydrazine derivatives of the

4   formula:

5
6 R_1
7                               $R - \overset{|}{N}-NH_2$

8   wherein R is a group of the formula

9
10
11                     $R_4 \diagdown \quad +$
12                          $N=C$
13                     $R_5 \diagup \quad \diagdown N-R_2$
14                                      $\overset{|}{R_3}$
15

16   and

17   R_1 is hydrogen or a lower alkyl group of 1-6 carbon

18   atoms, a hydroxyethyl group, or together with R_2 or R_4 may

19   be a lower alkylene bridge of 2-4 carbon atoms;

20   R_2 is hydrogen, amino, hydroxy, a lower alkyl group

21   of 1-6 carbon atoms, or together with R_1 or R_3 is a lower

22   alkylene bridge of 2-4 carbon atoms; R_2 may also be an

23   aminoalkylene group of the formula

76

$$-(CH_2)_n-\underset{\underset{R_7}{|}}{N}-R_6$$

27 wherein n is an integer of 2-7 and R_6 and R_7 are
28 independently a lower alkyl group of 1-6 carbon atoms or
29 together form a part of a cycloalkyl or heterocyclic ring
30 containing from 1 to 2 heteroatoms, of which at least one
31 is nitrogen; and the second of said heteroatoms is
32 selected from the group consisting of nitrogen, oxygen,
33 and sulfur; with the proviso that when the second of said
34 heteroatoms of the heterocyclic ring is nitrogen and
35 forms a piperazine ring; it may be optionally substituted
36 by a substituent that is identical to the portion of the
37 compound on the first nitrogen of the piperazine ring;
38   R_3 is hydrogen, a lower alkyl group of 1-6 carbon
39 atoms, or together with R_2 or R_4 is a lower alkylene
40 bridge of 2-4 carbon atoms;
41   R_4 is hydrogen, a lower alkyl group of 1-6 carbon
42 atoms or together with R_1 or R_3 is a lower alkylene bridge
43 of 2-4 carbon atoms; or an amino group;
44   R_5 is hydrogen, or a lower alkyl group of 1-6 carbon
45 atoms; with the proviso that at least one of R_1, R_2, R_3, R_4
46 or R_5 is other than hydrogen; or R is an acyl or a lower
47 alkylsulfonyl group of up to 10 carbon atoms and R_1 is
48 hydrogen; and their pharmaceutically acceptable acid
49 addition salts.

1 56. A method according to Claim 54 wherein the advanced
2 glycosylation endproduct-oxidized lipids are selected
3 from the group consisting of AGE-phospholipids, AGE-low
4 density lipoproteins, and AGE-apolipoproteins.

FIG. I
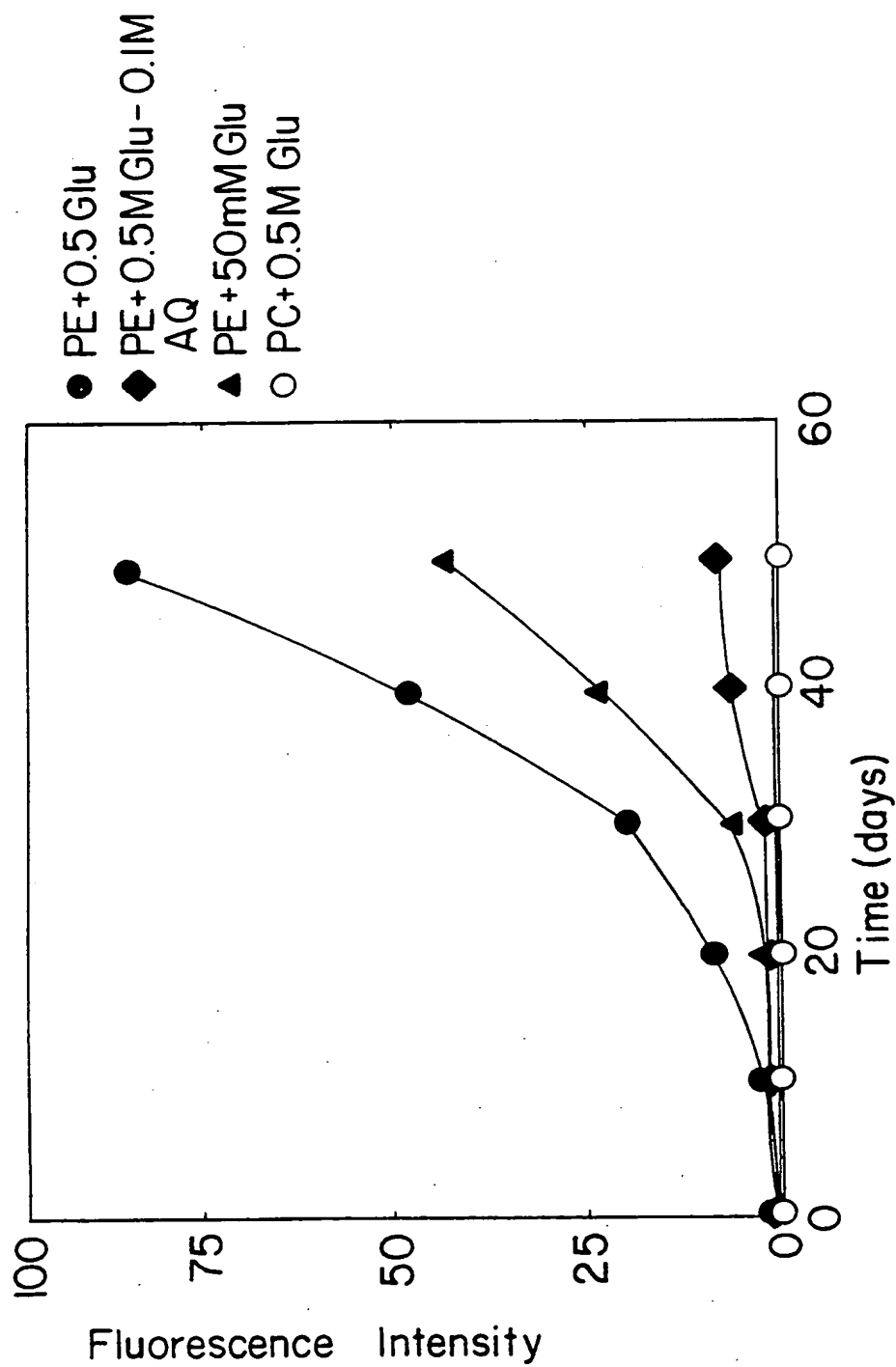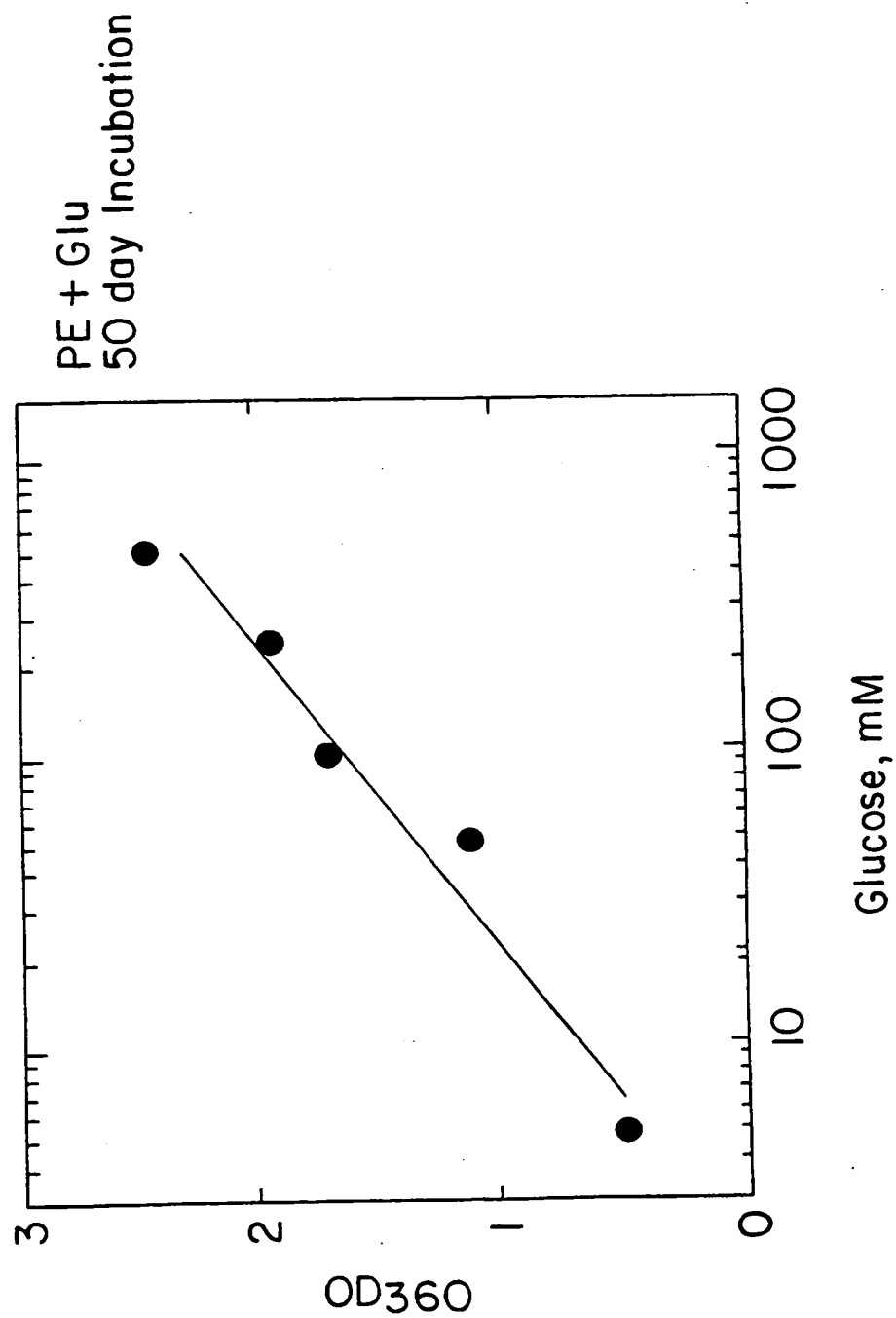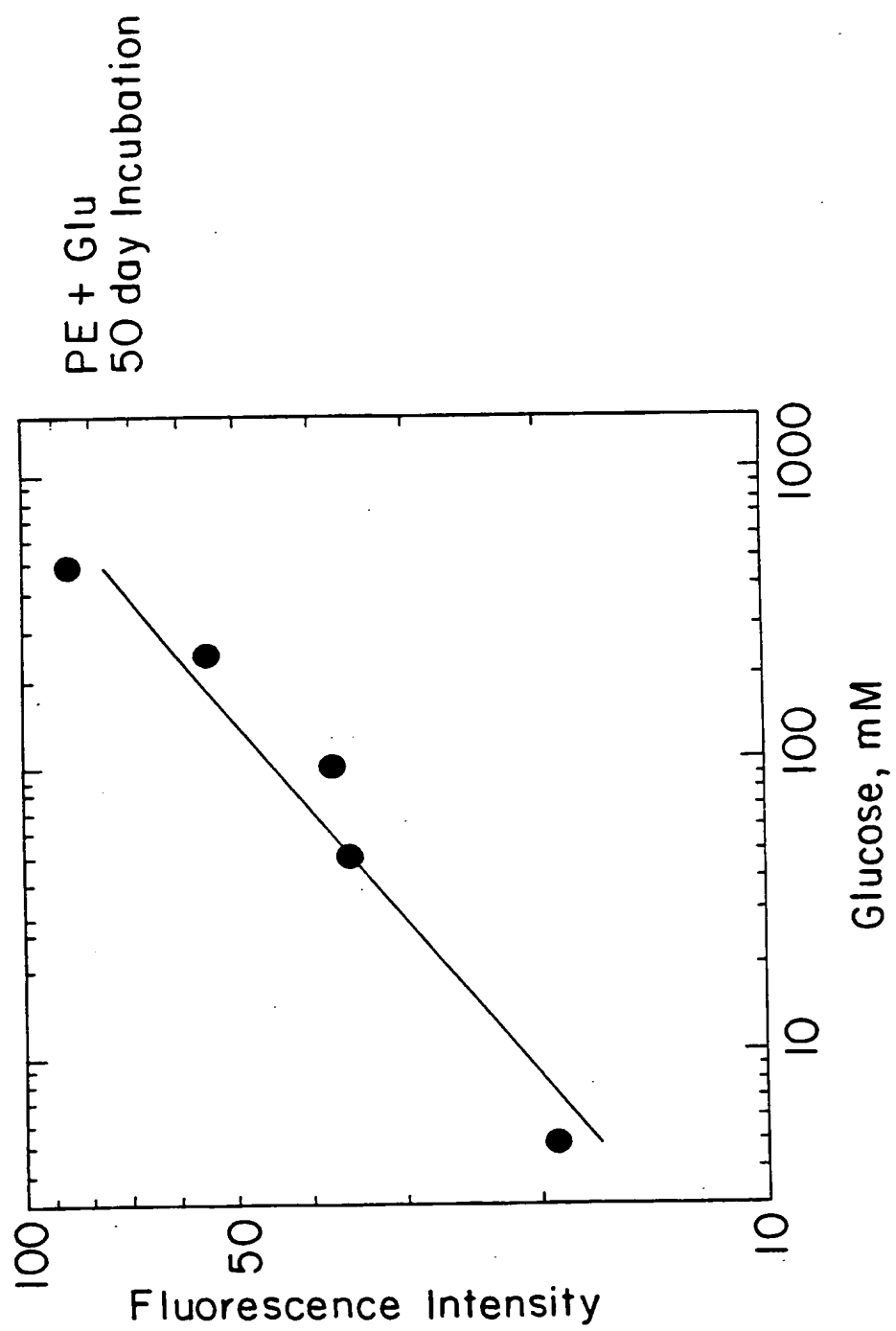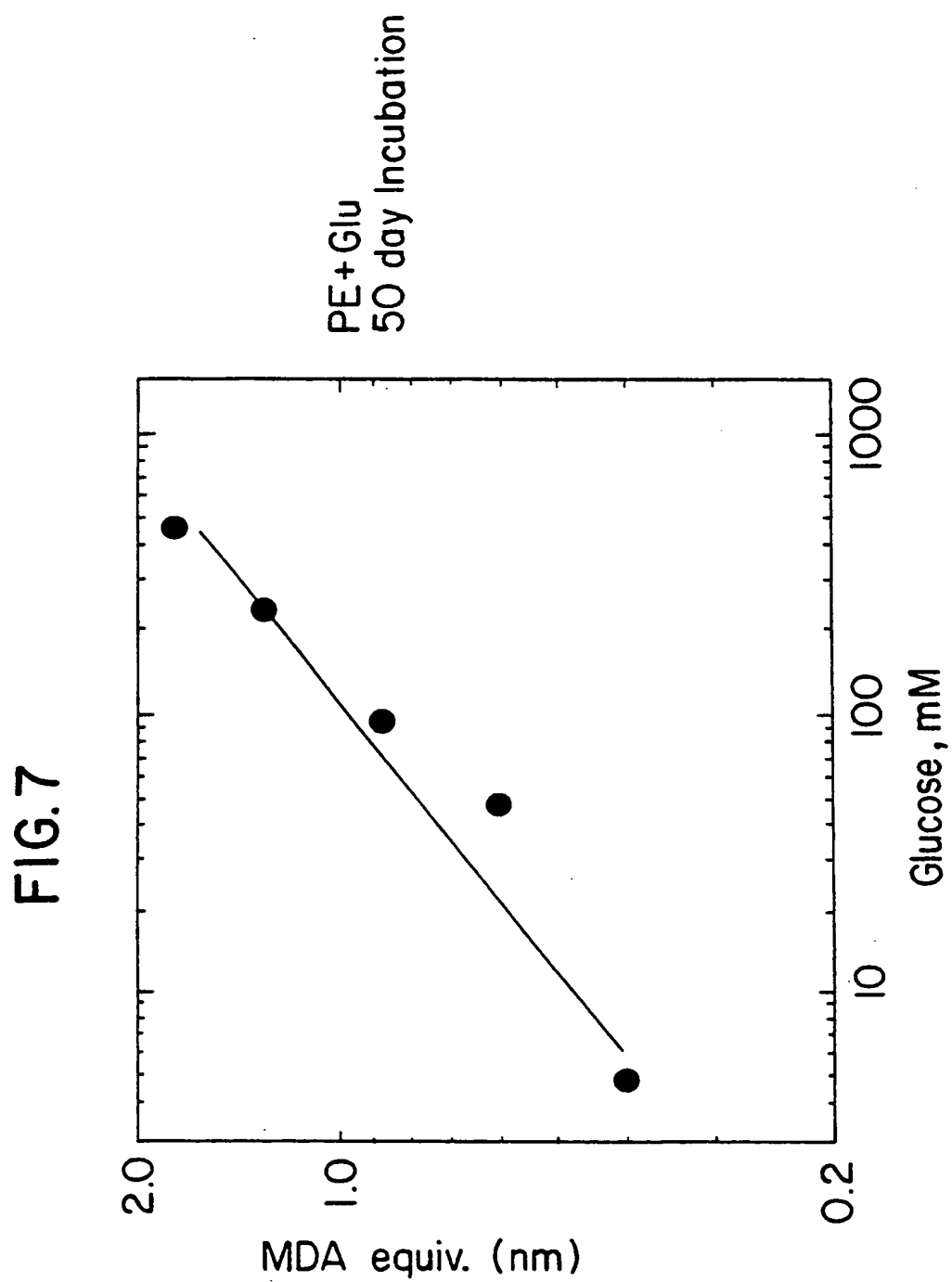
PE + 0.5 Glu
PE + 0.5 Glu + 0.1M AQ
PE + 50mM Glu
PC + 0.5M Glu

FIG.2

FIG.3

● PE + 0.5M Glu
◆ PE + 0.5M Glu + 0.1M AQ
▲ PE + 50mM Glu
○ PC + 0.5M Glu

FIG. 4

FIG. 5

PE + Glu
50 day Incubation

OD360

Glucose, mM

FIG.6

PE + Glu
50 day Incubation

FIG.7

PE+Glu
50 day Incubation

FIG. 8A

FIG.8B

FIG. 9C

pm MDA(eq)/μg LDL

FIG. 9B

U AGE/μg Lipid

FIG. 9A

U AGE/μg Apoprotein

FIG. IOA

FIG. IOB

FIG. IOC

FIG. IIB

FIG. IIA
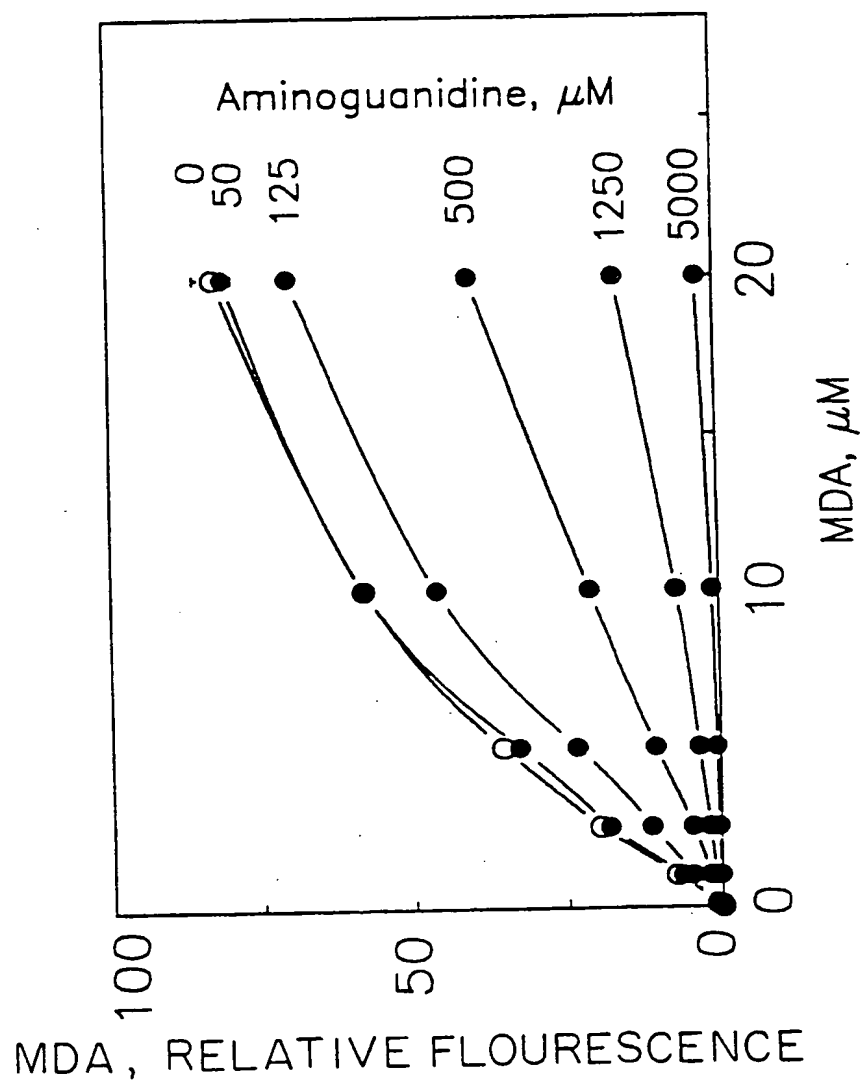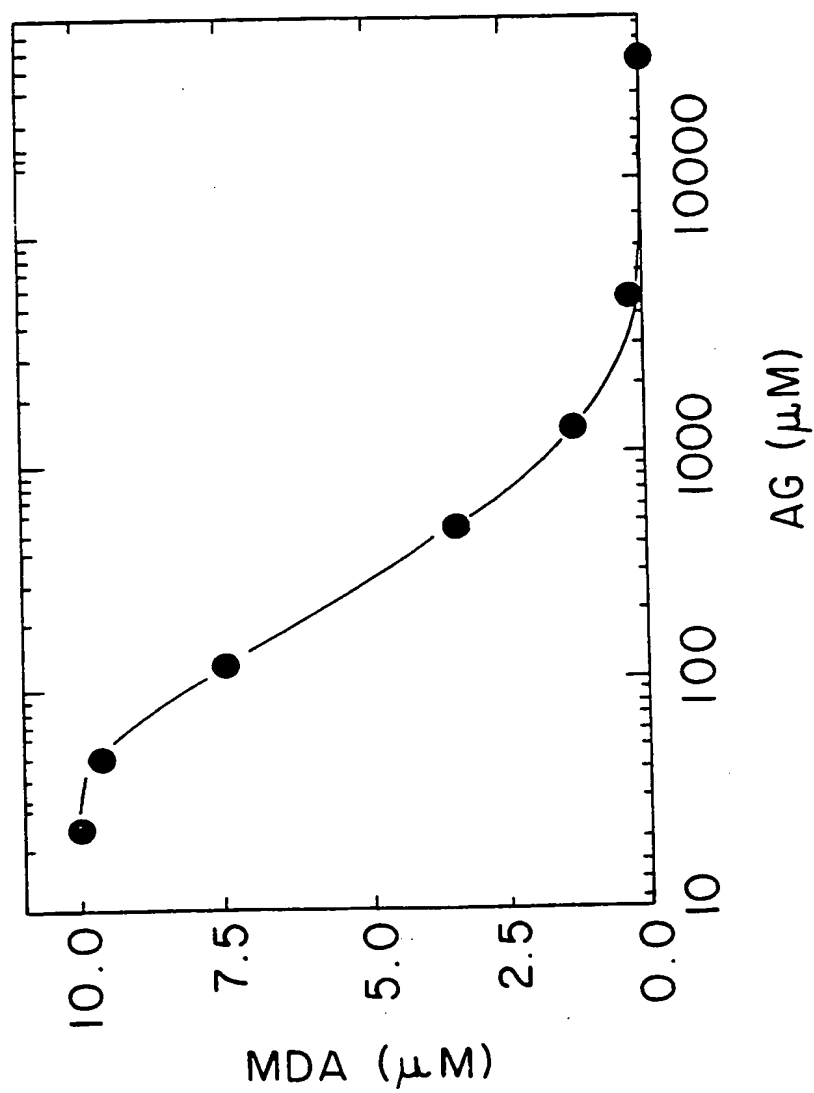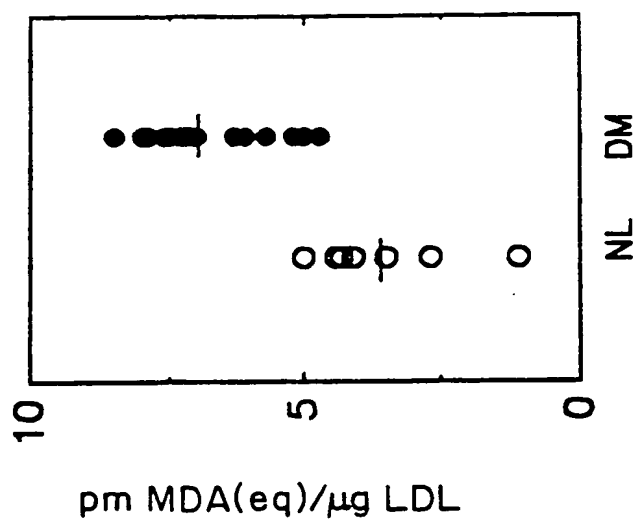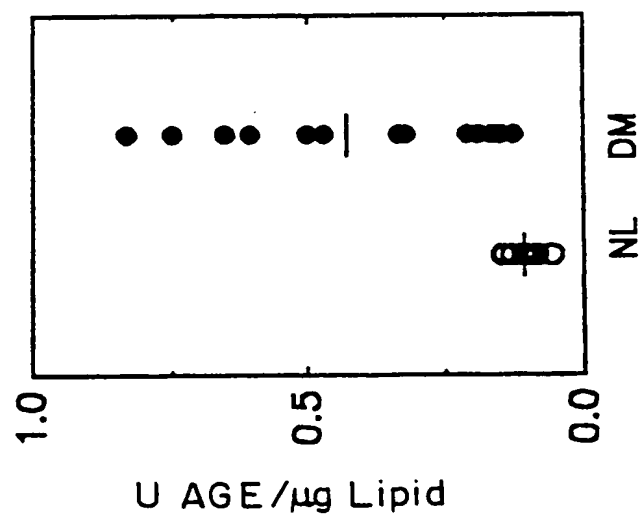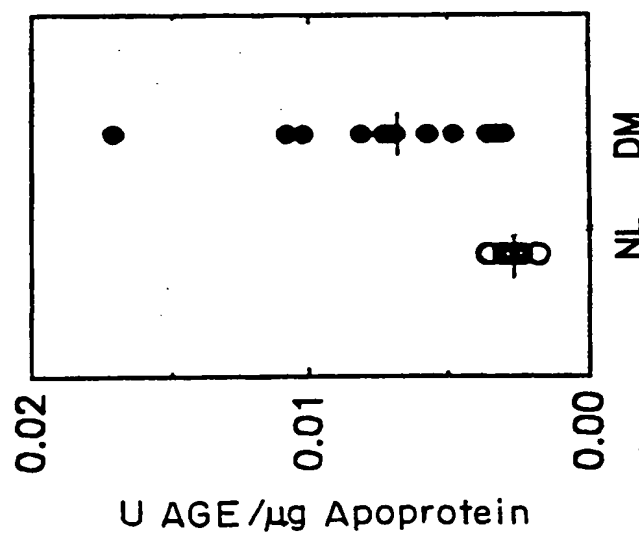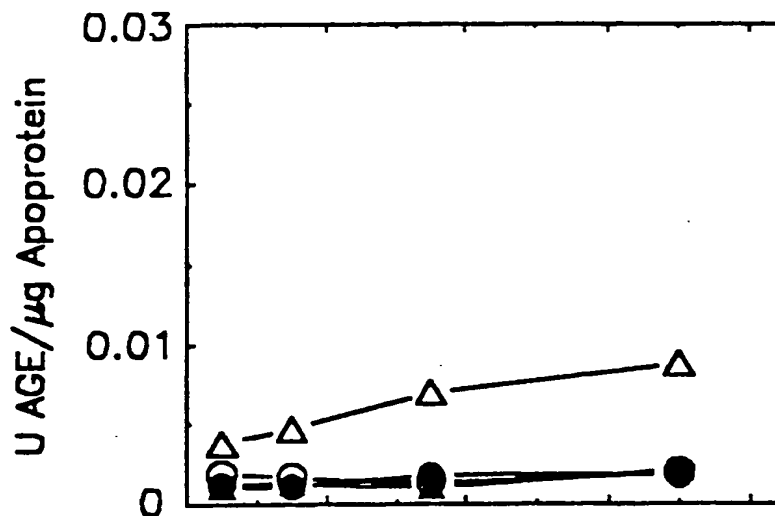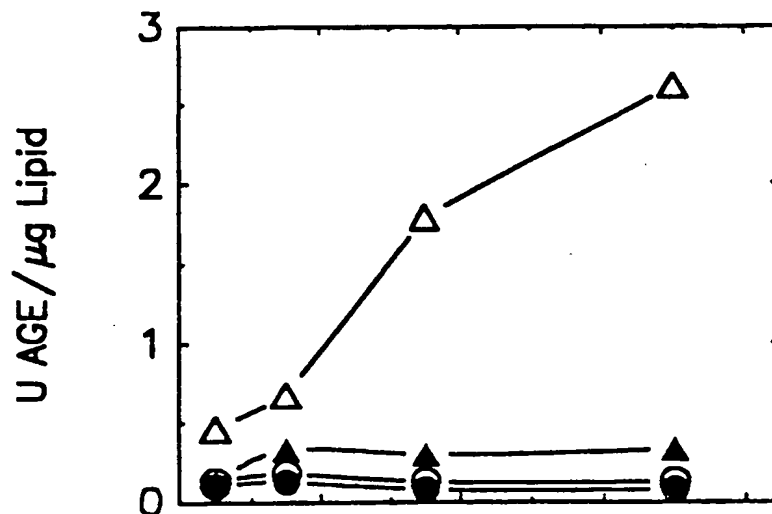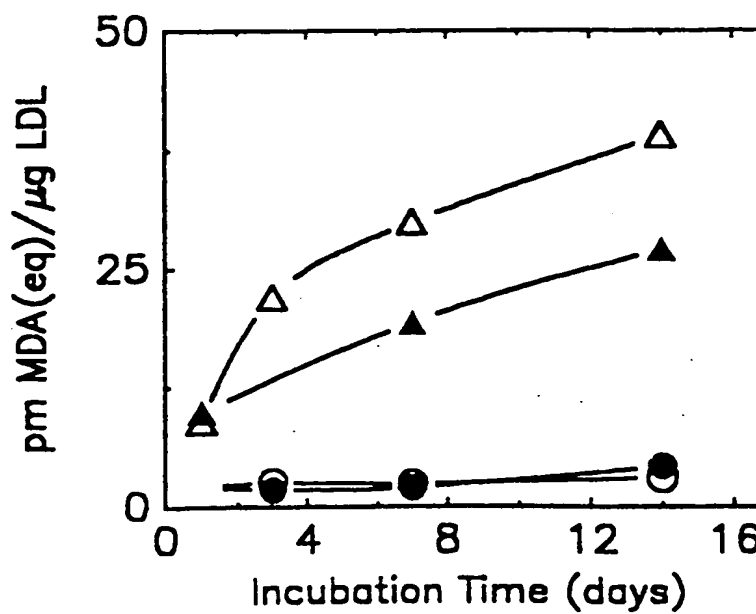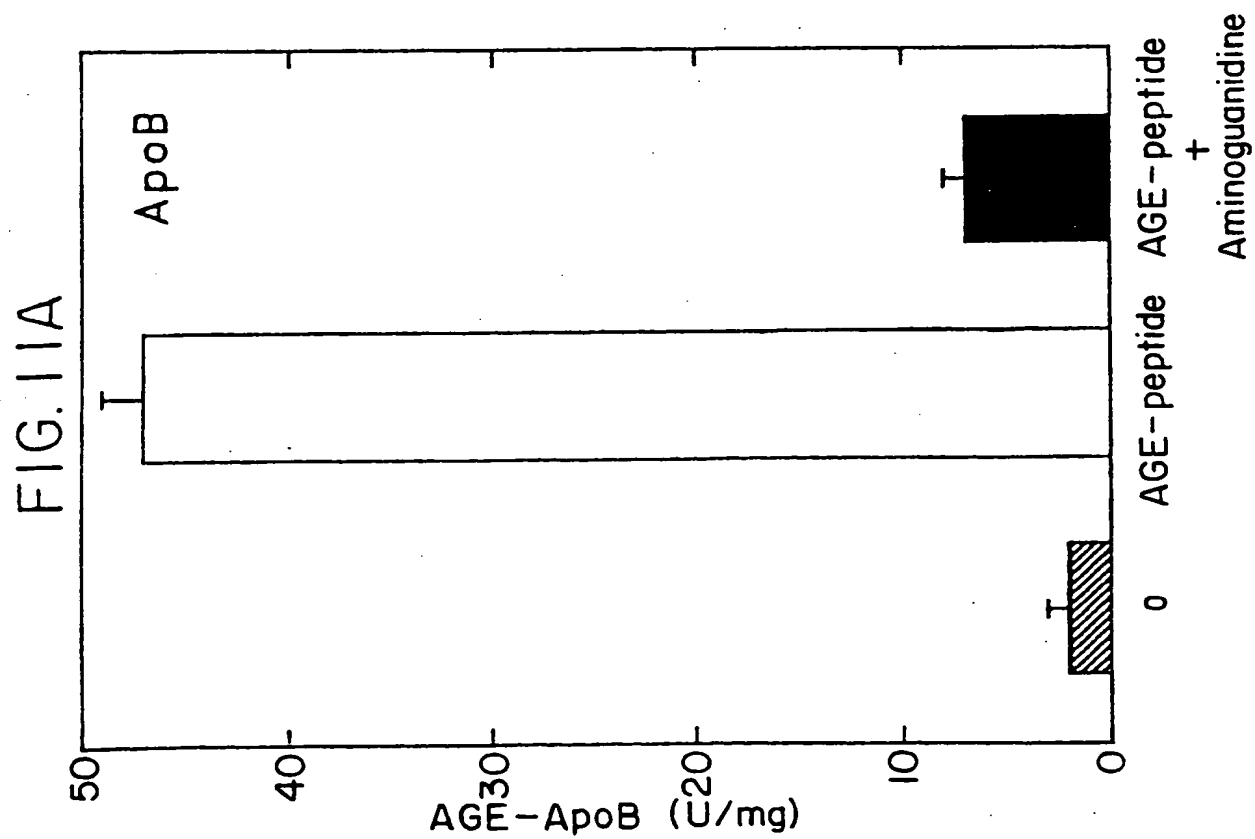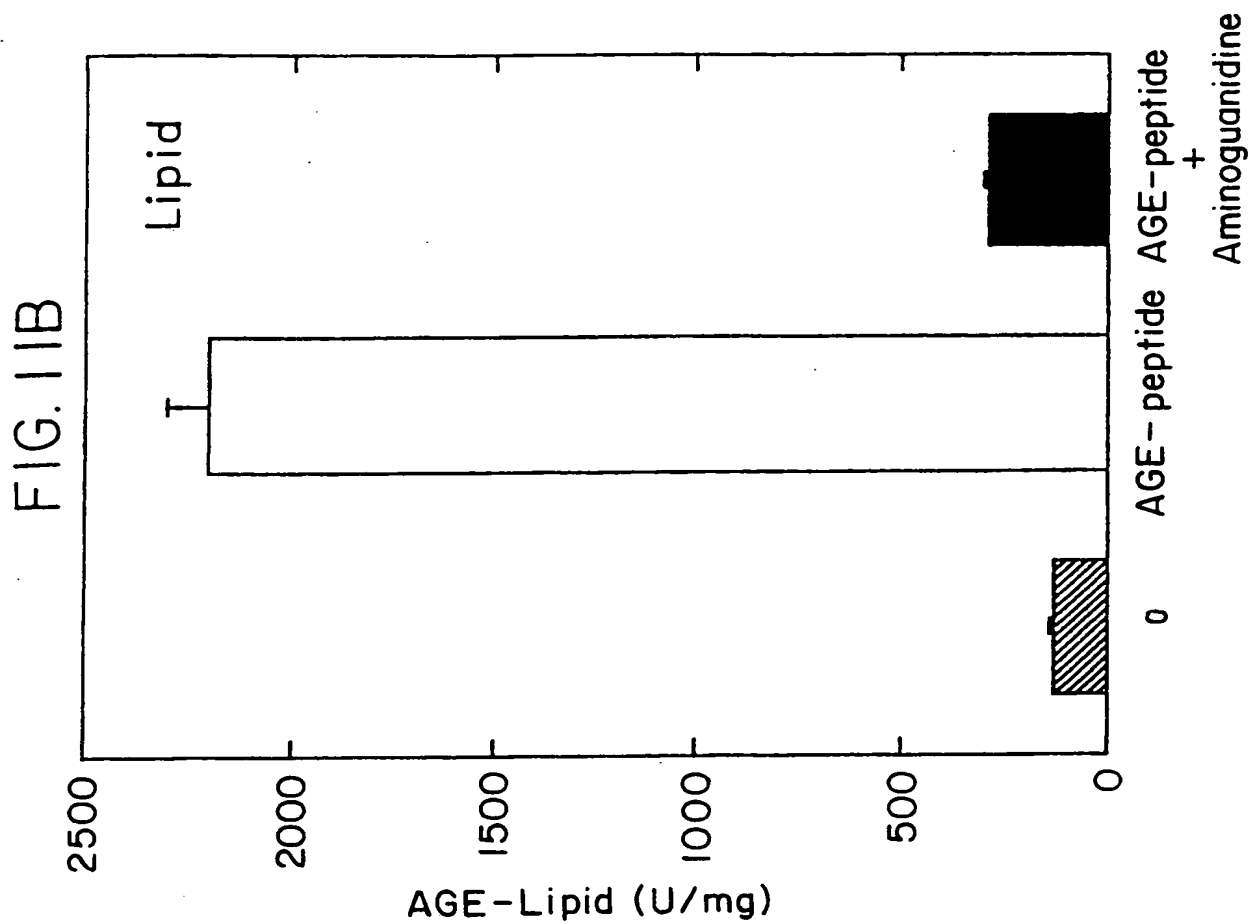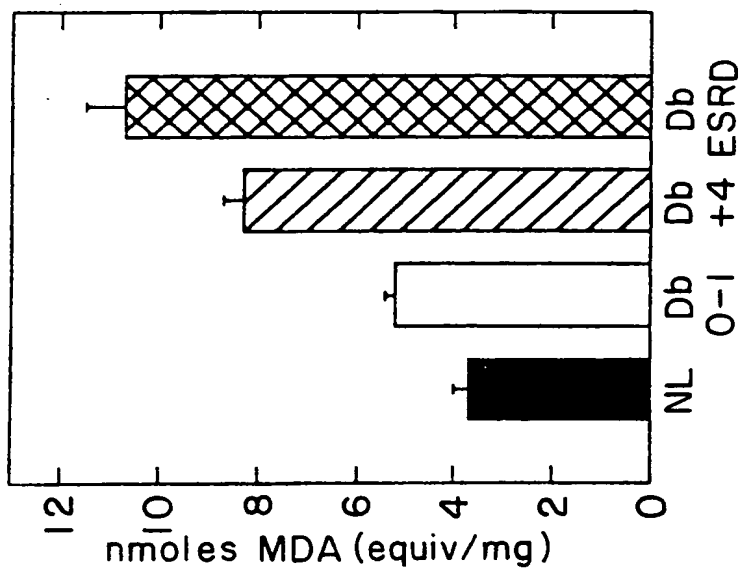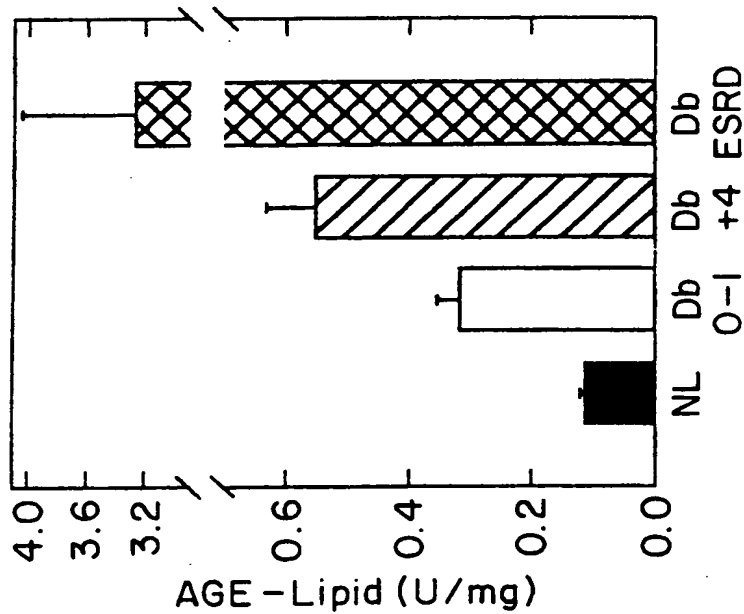
FIG.12C

FIG.12B

FIG.12A
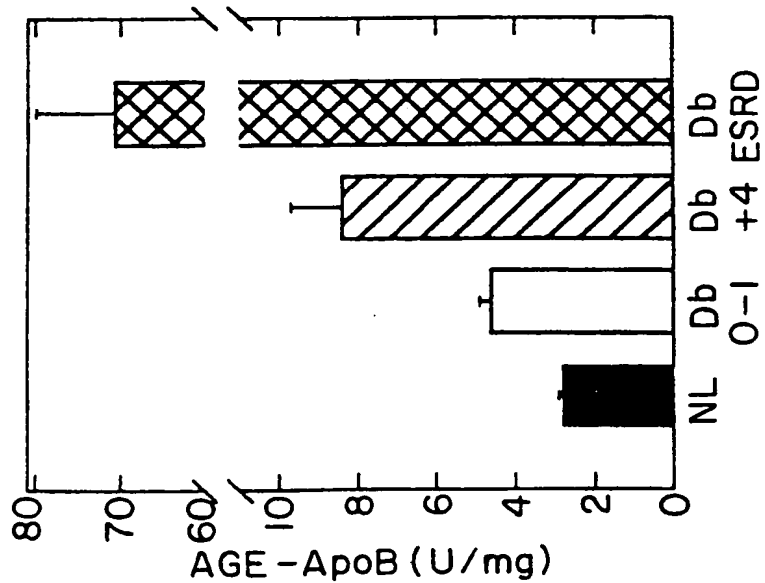
FIG. 13
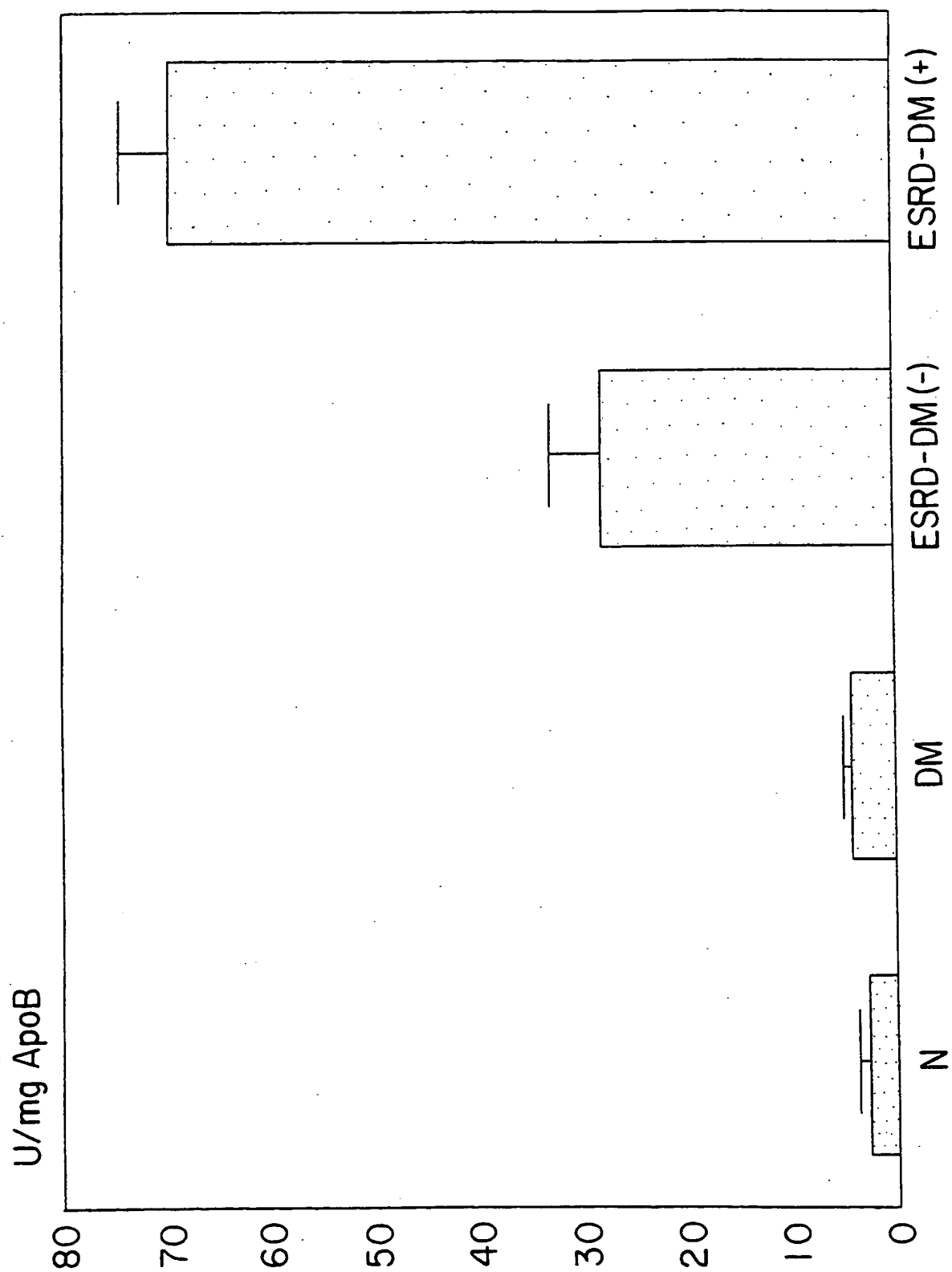
FIG.14
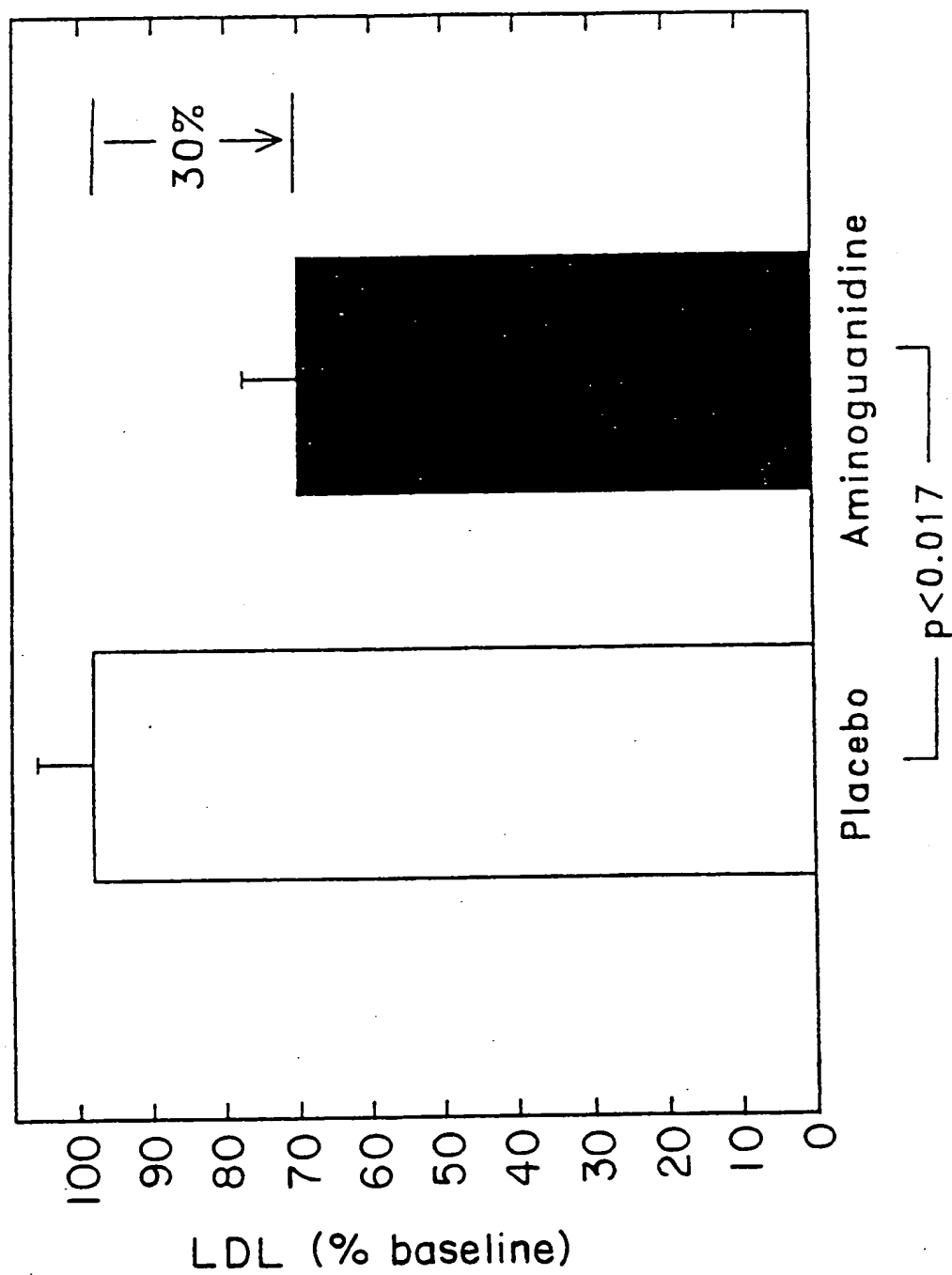
A. CLASSIFICATION OF SUBJECT MATTER
IPC 5   A61K31/155   A61K31/195   A61K31/415   A61K31/70   A61K35/00
G01N33/58   G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES vol. 90, no. 14 , 15 July 1993 pages 6434 - 6438 R. BUCALA ET AL. 'LIPID ADVANCED GLYCOSYLATION: PATHWAY FOR LIPID OXIDATION IN VIVO' see the whole document ---	1-15,20, 21, 25-34, 52-54,56
P,X	CLINICAL RESEARCH vol. 41, no. 2 , May 1993 page 183A R. BUCALA ET AL. 'INHIBITION OF ADVANCED GYLCOSYLATION BY AMINOGUANIDINE REDUCES PLASMA LDL LEVELS IN DIABETES' see the whole document --- -/--	1-13

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 1994	

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hoff, P

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,93 13421 (THE ROCKEFELLER UNIVERSITY) 8 July 1993 see abstract see page 16, line 12 - line 13 see page 22, line 11 - line 16; claims ---	46,47
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES vol. 89, no. 15 , 1992 pages 6876 - 6880 S. PICARD ET AL. 'AMINOGUANIDINE INHIBITS OXIDATIVE MODIFICATION OF LOW DENSITY LIPOPROTEIN PROTEIN AND THE SUBSEQUENT INCREASE IN UPTAKE BY MACROPHAGE SCAVENGER RECEPTORS' cited in the application	1-13,54, 56
Y	see the whole document ---	14,55
Y	EP,A,0 316 852 (THE ROCKEFELLER UNIVERSITY) 24 May 1989 cited in the application see abstract see page 6, line 48 - line 50 see page 9, line 53 - page 10, line 6; claims ---	14,55
A	DIABETE & METABOLISME vol. 17, no. 6 , 1991 pages 497 - 502 G. PONSIN ET AL. 'CONSEQUENCES METABOLIQUES DE LA GLUCOSYLATION NON ENZYMATIQUE DES APOLIPOPROTEINS'	1-27, 33-51, 54-56
X	see the whole document ---	28-32, 52,53
A	WO,A,93 04086 (THE ROCKEFELLER UNIVERSITY) 4 March 1993 see abstract; claims 1,26,31-45 ---	15-26
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 267, no. 8 , 1992 pages 5133 - 5138 Z. MAKITA ET AL. 'IMMUNOCHEMICAL DETECTION OF ADVANCED GLYCOSYLATION END PRODUCTS IN VIVO' cited in the application see the whole document ---	20-26

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE NEW ENGLAND JOURNAL OF MEDICINE vol. 318, no. 20 , 1988 pages 1315 - 1321 M. BROWNLEE ET AL. 'ADVANCED GLYCOSYLATION END PRODUCTS IN TISSUE AND THE BIOCHEMICAL BASIS OF DIABETIC COMPLICATIONS'	10-13, 15,28-32
A	see the whole document	1-9,14, 16-27, 33-56
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES vol. 87 , 1990 pages 9010 - 9014 M. KIRSTEIN ET AL. 'ADVANCED PROTEIN GLYCOSYLATION INDUCES TRANSENDOTHELIAL HUMAN MONOCYTE CHEMOTAXIS AND SECRETION OF PLATELET-DERIVED GROWTH FACTOR: ROLE IN VASCULAR DISEASE OF DIABETES AND AGING' see the whole document	28-34
X	DIABETOLOGIA vol. 30, no. 7 , 1987 page 568A G.P. PESCARMONA ET AL. 'NONENZYMATIC GLYCOSYLATION OF MEMBRANE PHOSPHOLIPIDS: A NEW MODEL OF CELLULAR DAMAGE IN INSULIN-INSENSITIVE TISSUES' see the whole document	28-32
X	EP,A,0 096 439 (UNILEVER) 21 December 1983 see abstract; claims	28-34
X	DIABETES vol. 34, no. 9 , 1985 pages 938 - 941 M. BROWNLEE ET AL. 'NONENZYMATIC GLYCOSYLATION PRODUCTS ON COLLAGEN COVALENTLY TRAP LOW-DENSITY LIPOPROTEIN'	28-34
Y	see the whole document	35-44, 49-51
Y	WO,A,85 04169 (THE ROCKEFELLER UNIVERSITY) 26 September 1985 see abstract; claims	49-51
Y	US,A,4 900 747 (VLASSARA ET AL.) 13 February 1990	35-44
X	see abstract see column 8, line 38 - column 9, line 22 see column 10, line 3 - line 7; claims	48

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-1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 222 313 (THE ROCKEFELLER UNIVERSITY) 20 May 1987 see the whole document -----	1-56

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:   X
because they relate to subject matter not required to be searched by this Authority, namely:
REMARK: Although claims 1-27, 40-44,46,47,52-56 are directed to a method of

treatment and to a method of diagnostic practised on the human/animal body
the search has been carried out and based on the alleged effects of the comPOUND/
                                                                                    COMPOSITION.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such
an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all
searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report
covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

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		AU-B-	622419	09-04-92
		JP-A-	2000156	05-01-90
		US-A-	4978684	18-12-90
		US-A-	5128122	07-07-92
		US-A-	5140048	18-08-92
		US-A-	5096703	17-03-92
		US-A-	5221683	22-06-93
		US-A-	5243071	07-09-93
		US-A-	5262152	16-11-93
		US-A-	5272165	21-12-93
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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